HOST-PATHOGEN INTERACTIONS INFLUENCING SUSCEPTIBILITY TO INFECTIVE ENDOCARDITIS

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DECLARATION

I declare that I have composed the thesis, the work detailed within the thesis is my own, and the work has not been submitted for any other degree or professional qualification.

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ABSTRACT

Introduction: Bacterial-platelet-fibrin complexes (vegetations), form on cardiac valves in infective endocarditis and are associated with increased morbidity and mortality. Although the mechanisms of bacterium-platelet adhesion, platelet activation and aggregation that are likely to contribute to vegetation formation have been identified, experimental conditions employed in these studies do not accurately reflect bacterial growth in the human vasculature. In addition, the contribution of host genetic factors such as platelet receptor polymorphisms to the pathogenesis of infective endocarditis is unknown. Considering that *Staphylococcus aureus* is now the most common cause of infective endocarditis associated with a poor prognosis, the contribution of bacterial and host factors to bacterium-platelet interactions, platelet activation and severity of infective endocarditis were analysed.

Methods and results:

Influence of bacterial growth environment on* S. aureus*-platelet interactions

Platelet aggregometry was performed with a range of *S. aureus* clinical and genetically modified isolates grown in nutrient broth and whole human blood. Some strains grown in nutrient broth failed to induce platelet aggregation, whereas all *S. aureus* isolates induced platelet aggregation after growth in blood. *S. aureus* surface proteins clumping factors A and B, serine-aspartate repeats C, D and E, iron-regulated surface determinants A and B and staphylococcal protein A were not essential for platelet aggregation induced by *S. aureus* grown in human blood, but the lag time to aggregation was increased in a strain containing mutations in genes encoding fibronectin-binding proteins A and B.

Correlation between platelet activation and susceptibility to infective endocarditis

Platelet activation was determined in patients with infective endocarditis using flow cytometry. Platelet P-selectin expression was reduced in patients with infective endocarditis as compared to healthy volunteers, but was higher in patients requiring surgery.

Influence of host genetic polymorphisms on* S. aureus*-platelet interactions and outcome in infective endocarditis

Flow cytometry and platelet aggregometry were performed to determine the role of specific platelet receptor GPIIIa $PI^{A1/A2}$, GPIb Kozak sequence, human platelet antigen (HPA)-2, variable number of tandem repeats (VNTR) and FcγRIIa H131R polymorphisms in *S. aureus*-platelet interactions. The GPIIIa $PI^{A1/A1}$ genotype, FcγRIIa H allele and GPIb Kozak sequence polymorphism were associated with increased *S. aureus*-induced platelet aggregation. GPIb VNTR alleles influenced aggregate formation *in vitro* and development of vegetations in patients with infective endocarditis. The GPIb HPA-2a/2a genotype was associated with increased incidence of the composite clinical end-point of embolism, heart failure, need for surgery and mortality in patients with infective endocarditis.

Conclusions: These studies have indicated that host and bacterial factors influence infective endocarditis and *S. aureus*-platelet interactions under conditions reflective of the host environment. Bacterial factors expressed during growth in human blood, host platelet activation levels and platelet receptor polymorphisms may represent novel prognostic markers and therapeutic targets in infective endocarditis.

LIST OF ABBREVIATIONS

ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
Agr	Accessory gene regulator
Arl	Autolysis-related locus
BHI	Brain-heart infusion
CA	Community-acquired
CC	Clonal complex
cDNA	Complementary DNA
CHIPS	Chemotaxis-inhibitory protein of staphylococci
CL	Chemically-defined metal limitation
ClfA	Clumping factor A
ClfB	Clumping factor B
Cna	Collagen adhesin
CRP	C-reactive protein
DIC	Disseminated intravascular coagulation
dNTP	Deoxynucleotide triphosphate
Eap	Extracellular adherence protein
ECG	Electrocardiograph
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
Efb	Extracellular fibrinogen-binding protein
EL	Erythrocyte-lysis
ELISA	Enzyme-linked immunosorbent assay
Emp	Extracellular matrix protein-binding protein
ERI	Edinburgh Royal Infirmary
FITC	Fluorescein isothiocyanate
FnBPA	Fibronectin-binding protein A

FnBPB	Fibronectin-binding protein B
Fur	Ferric-uptake repressor
GP	Glycoprotein
HA	Hospital-acquired
HPA	Human platelet antigen
ICAM	Inter-cellular adhesion molecule
Isd	Iron-regulated surface determinant
MHC	Major histocompatibility class
MI	Myocardial infarction
MLST	Multi-locus sequence typing
MRSA	Methicillin-resistant *S. aureus*
MSCRAMM	Microbial surface component recognising adhesive matrix molecules
MSSA	Methicillin-sensitive *S. aureus*
NEAT	NEAr Transporter
NHS	National Health Service
NTC	Non-template control
PAAP	Platelet aggregation-associated protein
PAR	Protease-activated receptor
PBP	Penicillin-binding protein
PBS	Phosphate-buffered saline
PCI	Percutaneous coronary intervention
PE	Phycoerythrin
Pls	Plasmin-sensitive protein
PMA	Platelet-monocyte aggregate
PMP	Platelet microbicidal protein
PPACK	D-Phenylalanyl-L-propyl-L-arginine chloromethylketone
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PSGL	P-selectin glycoprotein ligand
PVL	Panton-Valentine leucocidin
qRT-PCR	Quantitative reverse-transcriptase polymerase chain reaction

Rot	Repressor of toxins
RPMI	Roswell Park Memorial Institute
Sae	*S. aureus* exoprotein expression
Sar	Staphylococcal accessory regulator
Sas	*S. aureus* surface protein
SCCmec	Staphylococcal cassette chromosome
SCIN	Staphylococcal complement inhibitor
Sdr	Serine-aspartate repeat
SERAM	Secretable expanded repertoire adhesive molecules
SLV	Single locus variant
SNP	Single nucleotide polymorphism
SpA	Staphylococcal protein A
SraP	Serine-rich adhesin for platelets
SrpA	Serine-rich protein A
Srr	Staphylococcal respiratory response
SSL	Staphylococcal superantigen-like protein
ST	Sequence type
TE	TRIS-EDTA
TSA	Tryptic soy agar
TSST-1	Toxic shock syndrome toxin-1
VCAM	Vascular cell adhesion molecule
VISA	Vancomycin intermediate-resistant *S. aureus*
VNTR	Variable number of tandem repeat
VRSA	Vancomycin-resistant *S. aureus*
vWF	von Willebrand factor

CHAPTER 1

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1.1 Infections of the cardiovascular system

Recent evidence has suggested an association between infection and the development of a wide range of cardiovascular disorders. For example, a number of organisms including Coxsackie B, hepatitis C and adeno-viruses have been implicated in the pathogenesis of both myocarditis and pericarditis (Magnani & Dec, 2006). In addition, *Helicobacter pylori, Chlamydophila pneumoniae, Porphyromonas gingivalis,* herpes simplex virus and cytomegalovirus have been associated with the development of coronary artery disease (Kalvegren *et al.*, 2003, Vercellotti, 2001). However, in many cases there is no convincing evidence for an infective aetiology. A definitive link between infection and fatal cardiac disease is seen in infective endocarditis, a condition that develops following infection of the endocardium. The term infective endocarditis encompasses infection of native and prosthetic heart valves, implanted medical devices and large vessels (Horstkotte *et al.*, 2004).

1.2 Clinical aspects of infective endocarditis

1.2.1 Epidemiology of infective endocarditis

Infective endocarditis is a serious condition with an incidence of 2.43 to 9.29 per 100,000 person-years and predominantly affects men (Bashore *et al.*, 2006). Congestive cardiac failure is the most common complication, developing in up to 44% of patients, and is associated with an approximate two-fold increase in mortality (Heiro *et al.*, 2008, Murdoch *et al.*, 2009). Infective endocarditis may also be complicated by septic embolism in 21% to 34% of patients, with a resultant two- to three-fold increase in mortality (Chu *et al.*, 2004, Fabri *et al.*, 2006, Fowler *et al.*, 2005b, Heiro *et al.*, 2008, Thuny *et al.*, 2005). Between 0.3% to 3.3% of cases relapse and one or more recurrences occur in 6.6% to 12.3% of individuals (Heiro *et al.*, 2008, Mansur *et al.*, 2001). Despite current treatment options, the overall 1-year mortality rate of infective endocarditis has not changed in the past 30 years and

remains high at 12% to 31% (Habib *et al.*, 2009, Heiro *et al.*, 2008, Mansur *et al.*, 2001, Murdoch *et al.*, 2009).

It is already known that age, intravenous drug abuse and pre-existing valvular heart disease increase the risk of infective endocarditis (Bashore *et al.*, 2006). However, 14% of patients with infective endocarditis have no underlying risk factors, and this increases to over 50% in those with *Staphylococcus aureus* infective endocarditis (Fowler *et al.*, 1999, Murdoch *et al.*, 2009, Netzer *et al.*, 2000). Considering the high morbidity and mortality associated with infective endocarditis, it is important to elucidate why it occurs as a complication of bacteraemia in some cases, but not others.

1.2.2 The changing face of infective endocarditis

Recent articles have alluded to the 'changing face' of infective endocarditis in reference to the shifting epidemiology of this condition (Mouly *et al.*, 2002, Prendergast, 2006). Although viridans streptococci including *Streptococcus sanguinis*, *Streptococcus oralis* and *Streptococcus mitis* have traditionally been the most common cause of infective endocarditis, *S. aureus* now accounts for up to 49% of cases in non-intravenous drug abusers, in comparison to 14% to 21% for viridans streptococci (Table 1.1) (Moreillon & Que, 2004, Murdoch *et al.*, 2009, Watanakunakorn & Burkert, 1993).

Table 1.1. Causes of infective endocarditis classified by patient sub-group. Data adapted from (Moreillon & Que, 2004).

Pathogen	Native valve infective endocarditis	Infective endocarditis in IVDAs	Prosthetic valve infective endocarditis
Staphylococci	44%	69%	49%
S. aureus	38%	69%	21%
Coagulase-negative staphylococci	6%	0%	28%
Streptococci	31%	8%	29%
Oral streptococci	21%	3%	22%
Others	10%	5%	7%
Enterococci	8%	2%	7%
HACEK group	4%	0%	1%
Other bacteria	4%	5%	2%
Fungi	1%	2%	0%
Polymicrobial	2%	9%	1%
Negative blood cultures	6%	5%	10%

IVDA, intravenous drug abusers; HACEK, *Haemophilus* spp, *Aggregatibacter actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens* and *Kingella kingae*.

The emergence of *S. aureus* as the main cause of infective endocarditis is largely through iatrogenic means (Fowler *et al.*, 2005b). Long-term intravenous or intra-arterial access routes, intra-cardiac devices and prostheses act as portals of entry for bacteria (Fowler *et al.*, 2005b, Moreillon *et al.*, 2002). Commensal bacteria such as *S. aureus* that normally colonise human skin with no adverse effects are able to gain access to the bloodstream and persist on cardiac valves. Furthermore, there has been an increase in the incidence of community-acquired *S. aureus* infective endocarditis due to the increased prevalence of haemodialysis catheters, pacemakers and prosthetic heart valves in the community (Millar *et al.*, 2008). Intravenous drug abuse, an ageing population and improved diagnostic procedures have also contributed to the increased prevalence of *S. aureus* infective endocarditis (Moreillon *et al.*, 2002, Nadji *et al.*, 2005).

S. aureus has been associated with a more aggressive course of infective endocarditis as compared to other organisms such as streptococci, manifested by increased rates of emboli and death (Fowler *et al.*, 2005b, Nadji *et al.*, 2005). This may be due in part to its ability to colonise undamaged human heart valves (Moreillon *et al.*, 2002). Despite advances in diagnostics and therapeutics, the mortality rate of *S. aureus* infective endocarditis approaches 65%, which is higher than that observed for most other organisms (Cabell *et al.*, 2002, Fowler *et al.*, 2005b, Nadji *et al.*, 2005).

1.2.3 Diagnosis of infective endocarditis

The diagnosis of infective endocarditis is largely based upon the modified Duke criteria, where positive serial blood cultures for typical microorganisms and echocardiographic features form the main criteria (Table 1.2) (Li *et al.*, 2000b). Infective endocarditis is considered to be definite in the presence of 1 pathological Duke criterion, 2 major clinical criteria, 1 major and 3 minor clinical criteria or all 5 minor criteria, and is thought to be possible when 1 major and 1 minor clinical criterion or 3 minor clinical criteria are fulfilled (Li *et al.*, 2000b). The diagnosis of infective endocarditis is rejected if the criteria are not met, if there is a firm

alternative diagnosis, or if the patient's symptoms resolve within 4 d of antibiotic therapy (Li *et al.*, 2000b). Clinical judgement is often used to aid diagnosis.

There have been conflicting results from clinical studies regarding the type of echocardiogram that should be performed to safely exclude infective endocarditis. Fowler *et al* identified increased sensitivity and specificity with transoesophageal echocardiography in the detection of vegetations, particularly in patients with *S. aureus* bacteraemia, prosthetic valves, abscesses or right-sided involvement, and advocate its use in all patients (Fowler *et al.*, 1997). However, other studies have suggested that only those with a high pre-test probability of infective endocarditis, as evidenced by embolic phenomena or significant valvular regurgitation on transthoracic echocardiography, should have a transoesophageal echocardiogram (Thangaroopan & Choy, 2005, Van Hal *et al.*, 2005). Although transoesophageal echocardiography is more invasive than transthoracic echocardiography, it appears to be better overall at excluding infective endocarditis.

Table 1.2. Modified Duke criteria for the diagnosis of infective endocarditis. Table adapted from (Li *et al.*, 2000b).

Type of criteria	Features
Pathological Criteria	1 Microorganisms demonstrated by culture or histological examination of a vegetation, a vegetation that has embolised, or an intracardiac abscess specimen, or
	2 Pathological lesions - vegetation or intracardiac abscess confirmed by histological examination showing active endocarditis
Major Clinical Criteria	1. Blood culture positive for infective endocarditis
	a) Typical microorganisms consistent with infective endocarditis from 2 separate blood cultures:
	i. Viridans streptococci, *Streptococcus bovis*, HACEK group, *Staphylococcus aureus*, or
	ii. Community-acquired enterococci, in the absence of a primary focus, or
	b) Microorganisms consistent with infective endocarditis from persistently positive blood cultures, defined as follows:
	i. At least 2 positive cultures of blood samples drawn >12 h apart, or
	ii. All of 3 or a majority of 4 separate cultures of blood (with first and last sample drawn at least 1 h apart)

Type of criteria	Features
	c) Single positive blood culture for *Coxiella burnetii* or antiphase I IgG antibody titre > 1:800
	2. Evidence of endocardial involvement
	3. Echocardiogram positive for infective endocarditis a) Oscillating intracardiac mass on valve or supporting structures, in the path of regurgitant jets, or on implanted material in the absence of an alternative anatomic explanation, or b) Abscess, or c) New partial dehiscence of prosthetic valve
	4. New valvular regurgitation (worsening or changing of pre-existing murmur not sufficient)
Minor Clinical Criteria	1. Predisposition, predisposing heart condition or injection drug use
	2. Fever, temperature >38°C
	3. Vascular phenomena: major arterial emboli, septic pulmonary infarcts, mycotic aneurysms, intracranial haemorrhage, conjunctival haemorrhages, and Janeway's lesions
	4. Immunologic phenomena: glomerulonephritis, Osler's nodes, Roth's spots, and rheumatoid factor

Type of criteria	Features
	5. Microbiological evidence: positive blood culture but does not meet a major criterion as noted above or serological evidence of active infection with organism consistent with infective endocarditis

HACEK, *Haemophilus* spp, *Aggregatibacter actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens* and *Kingella kingae*.

1.2.4 Management and prevention of infective endocarditis

Current treatment of infective endocarditis follows national and international guidelines, such as those issued by the European Society of Cardiology, and includes the use of appropriate antibiotics according to microbiological culture and sensitivity results (Habib *et al.*, 2009). However, approximately 40% of cases fail to respond to medical therapy alone and require valve replacement or repair (Cabell *et al.*, 2005).

Surgical treatment of infective endocarditis is often delayed until the acute phase of infection has resolved, in order to minimise the risk of peri- and early post-operative infective endocarditis (Habib *et al.*, 2009). However, early surgery is indicated for patients at high risk of developing intractable cardiac failure, those with large vegetations with potential for embolism, abscesses, fistulae, valve obstruction, destruction or dehiscence and infection with organisms that are refractory to antibiotic treatment (Bashore *et al.*, 2006, Habib *et al.*, 2009).

Surgical treatment is associated with a significant improvement in prognosis, especially in patients with aortic valve or *S. aureus* infective endocarditis (Bishara *et al.*, 2001, Cabell *et al.*, 2005, Croft *et al.*, 1983), but these trials are predominantly retrospective, single-centre observational studies (Bishara *et al.*, 2001, Croft *et al.*, 1983, Habib *et al.*, 2009). Furthermore, the potential benefits of surgery in the most seriously ill patients are unknown, as they are often deemed to have unacceptably high risks of peri-operative complications, including death (Habib *et al.*, 2009).

Antibiotic prophylaxis has traditionally been recommended for patients considered to be at risk of infective endocarditis, including those with acquired or congenital structural heart disease, prosthetic heart valves and previous infective endocarditis, undergoing invasive dental, genitourinary and gastrointestinal procedures (Habib *et al.*, 2009). However, no randomised controlled trials have analysed the potential benefits of oral antibiotic prophylaxis, whereas it is widely known that antimicrobials such as penicillins are associated with a risk of anaphylaxis (Habib et al., 2009). Recent guidelines issued by the National Institute for Clinical Excellence and the

European Society of Cardiology have revised recommendations for antibiotic prophylaxis, such that only high-risk patients undergoing high-risk procedures are advised to receive preventative treatment (Habib et al., 2009, National Institute for Health and Clinical Excellence, 2008). It is clear that more robust evidence is required to determine the benefits of existing and novel therapeutics and prophylactics in infective endocarditis.

1.3 *Staphylococcus aureus*, the major infective endocarditis pathogen

1.3.1 Diseases caused by *S. aureus*

S. aureus is a coagulase-positive, Gram-positive coccus (Lowy, 1998). First identified by Sir Alexander Ogston in Aberdeen, Scotland, 1880, *S. aureus* is the most pathogenic human staphylococcal species, responsible for a wide array of invasive and non-invasive diseases (Table 1.3) (van Belkum *et al.*, 2009). Endocarditis currently accounts for 18% to 35% of *S. aureus* bacteraemia cases (Fowler *et al.*, 1997, Lowy, 1998, Van Hal *et al.*, 2005), increasing to 51% in patients with a prosthetic heart valve (El-Ahdab *et al.*, 2005). *S. aureus* has also been implicated in a variety of infectious diseases in animals, ranging from mastitis in ruminants and skeletal infections in chickens, to skin and bloodstream infections in rabbits (Ben Zakour *et al.*, 2008).

Table 1.3. The wide range of human diseases caused by *S. aureus* (Lowy, 1998, Greene *et al.*, 1995, Sinha & Herrmann, 2005, van Belkum *et al.*, 2009).

Human (non-invasive)	Human (invasive)
Boils	Cystitis
Carbuncles	Gastroenteritis
Cellulitis	Indwelling catheter sepsis
Folliculitis	Infective endocarditis
Furuncles	Meningitis
Impetigo	Osteomyelitis
Sinusitis	Pneumonia
Staphylococcal scalded skin syndrome	Septic arthritis
Stye	Septicaemia
Superficial abscesses	Toxic shock syndrome
	Urinary tract infections
	Wound infections/deep abscesses

S. aureus colonises the human nares, axillae, pharynx, vagina and skin, with a combination of host, bacterial and environmental factors contributing to host colonisation (van Belkum et al., 2009, Wertheim *et al.*, 2005a). The anterior nares are the most common site of colonisation in humans, with approximately 20% of healthy adults permanently colonised and 30% intermittently colonised by *S. aureus* (Lowy, 1998, Wertheim et al., 2005a). Permanent carriers are thought to constantly harbour the same strain, while intermittent carriers tend to be colonised by different strains (Wertheim et al., 2005a). In the presence of favourable conditions, commensal isolates have the potential to cause invasive infection, but the underlying virulence and host factors contributing to this are not fully understood (Feil *et al.*, 2003, Lindsay *et al.*, 2006, Wertheim et al., 2005a).

1.3.2 Antibiotic resistance in *S. aureus*

With the increased number of infections attributable to *S. aureus*, there has been an associated increase in antimicrobial use, leading to antibiotic resistance (Lowy, 2003). Prior to the advent of the β-lactam antibiotic penicillin, 70% of *S. aureus* infections were complicated by haematogenous dissemination with an associated 80% mortality rate (Deurenberg *et al.*, 2007, Lowy, 2003). The introduction of penicillin in clinical practice in 1940 substantially reduced *S. aureus*-associated morbidity and mortality, but was followed 2 years later by the first case of penicillin-resistance due to the acquisition of β-lactamases by *S. aureus* (Deurenberg & Stobberingh, 2008, Lowy, 2003). Within 10 years, approximately 90% of *S. aureus* strains had developed penicillin-resistance (Boyle-Vavra & Daum, 2007, Lowy, 2003).

The β-lactamase-resistant antibiotic methicillin, was introduced in 1959, but was swiftly followed by the first case of methicillin-resistant *S. aureus* (MRSA), identified in the United Kingdom in 1961 (Deurenberg & Stobberingh, 2008). Since then, the number of cases of MRSA has escalated worldwide, particularly in the context of nosocomial infection, with associated high mortality rates (Boyle-Vavra &

Daum, 2007, Fowler *et al.*, 2005b, Lowy, 2003). Approximately 27% of cases of *S. aureus* bacteraemia or infective endocarditis in developed countries are due to MRSA (Fowler *et al.*, 2005b).

Methicillin resistance is conferred by the acquisition of penicillin-binding protein 2a (PBP2a) encoded by the *mecA* gene on staphylococcal cassette chromosome (SCC*mec*) (Boyle-Vavra & Daum, 2007). PBP2a prevents binding of β-lactams to *S. aureus*, enabling *S. aureus* cell wall synthesis to continue and facilitating pathogen survival (Boyle-Vavra & Daum, 2007). Seven different types of SCC*mec* have been identified to date and are classified according to the type of recombinase they encode (Deurenberg & Stobberingh, 2008).

MRSA has traditionally been associated with hospital stay, but community-acquired MRSA (CA-MRSA) is increasing in prevalence (Deurenberg *et al.*, 2007). CA-MRSA first emerged in the 1990s and is now a global phenomenon (Diep & Otto, 2008). As of 2007, there were 23 published cases of CA-MRSA infective endocarditis (Millar *et al.*, 2008).

CA-MRSA differs from hospital-acquired MRSA (HA-MRSA) in that younger individuals with a history of skin lesions, intravenous drug abuse or diabetes mellitus are more likely to be affected (Millar *et al.*, 2008). Furthermore, CA-MRSA is more likely to harbour SCC*mec* types IV or V, while HA-MRSA possesses SCC*mec* types I to III (Deurenberg *et al.*, 2007). Notably, SCC*mec* IV is smaller, more likely to be horizontally transferred and contains fewer antibiotic resistance genes than SCC*mec* types I to III (Deurenberg *et al.*, 2007). CA- and HA-MRSA strains also have different genetic backgrounds (see Section 1.3.3), leading to the belief that CA-MRSA originated from CA-methicillin sensitive *S. aureus* (MSSA) rather than HA-MRSA strains (Boyle-Vavra & Daum, 2007).

CA-MRSA strains, particularly those of the USA300 lineage, have been associated with increased virulence as compared to HA-MRSA strains (Li *et al.*, 2009). The increased virulence of CA-MRSA was initially attributed to Panton-Valentine leucocidin (PVL), a bi-component pore-forming leucotoxin present in a large

proportion of CA-MRSA isolates (Boyle-Vavra & Daum, 2007, Labandeira-Rey *et al.*, 2007). Although an early study identified an association between the presence of PVL and the development of necrotising pneumonia in a murine model of infection (Labandeira-Rey *et al.*, 2007), recent evidence suggests that the creation of an isogenic PVL-negative mutant in this study was complicated by an unintentional point mutation in the P2 promoter of the accessory gene regulator (*agr*) (see Section 1.3.6) (Villaruz *et al.*, 2009). Subsequent repair of the *agr* mutation resulted in PVL having no significant effect on the pathogenesis of severe pneumonia (Villaruz *et al.*, 2009). Recently, the pathogenicity of CA-MRSA has been attributed to increased production of core genome-encoded virulence factors such as α-toxin and phenol-soluble modulins (Li *et al.*, 2009).

Some MRSA strains have now acquired reduced susceptibility to the 'antibiotic of last resort' vancomycin, resulting in the emergence of vancomycin intermediate-resistant *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA) (Lowy, 2003). VISA, which first emerged in Japan in 1997, develops following changes in *S. aureus* peptidoglycan synthesis and cross-linking, leading to thickened, irregular cell walls that trap vancomycin (Lowy, 2003). Conversely, complete resistance to vancomycin first occurred in 2002 following transfer of the *vanA* operon from vancomycin-resistant enterococci, resulting in alteration of the terminal peptide of peptidoglycan, leading to reduced affinity for vancomycin (Lowy, 2003). At present 29% of cases of MRSA infective endocarditis are due to strains that are also VISA, and such cases are associated with increased rates of persistent bacteraemia and cardiac failure (Bae *et al.*, 2009).

Daptomycin has recently emerged as an effective antibiotic for the treatment of MRSA, VISA and VRSA infections (Fowler *et al.*, 2006). However, cases of reduced susceptibility to daptomycin, often linked with vancomycin resistance, have arisen (Kirby *et al.*, 2009, Sakoulas *et al.*, 2008). It is clear that new therapeutics are required in the management of *S. aureus* bacteraemia and infective endocarditis.

1.3.3 Population genetics and evolution of *S. aureus*

The *S. aureus* genome varies in size from a 2.5 to 2.9 Mbp circular chromosome that codes for numerous virulence factors and may also contain antibiotic resistance genes (Lowy, 1998, Sinha & Herrmann, 2005). Since the *S. aureus* genome was first sequenced in 2001, 15 *S. aureus* strain sequences have been published, with over 100 strains currently being sequenced (National Centre for Biotechnology Information, National Centre for Biotechnology Information, UniProt Consortium). The *S. aureus* genome is considered to consist of the core, core variable and accessory genomes (Lindsay *et al.*, 2006). Three-quarters of genes are present in the core genome, which is largely invariable as the genes present are involved in so-called 'housekeeping' functions, which facilitate *S. aureus* growth and survival (Ben Zakour *et al.*, 2008). Approximately 10% to 12% of genes are in the core variable genome, including many virulence factors (Ben Zakour *et al.*, 2008, Lindsay *et al.*, 2006). The accessory genome predominantly contains horizontally-acquired genes encoding antibiotic resistance and virulence factors (Ben Zakour *et al.*, 2008). Mobile genetic elements such as genomic islands, staphylococcal pathogenicity islands, prophages, integrated plasmids and transposons make up the accessory genome and confer marked plasticity to the *S. aureus* genome (Lindsay *et al.*, 2006, Peacock *et al.*, 2002).

The clonal diversity of *S. aureus* has been examined by multi-locus sequence typing (MLST) (Feil *et al.*, 2003). A study by Feil *et al* demonstrated that 78% of *S. aureus* strains could be grouped into 1 of 11 clonal complexes (CC), consisting of *S. aureus* sequence type (ST) strains and single locus variants (SLVs), the latter only differing from STs at 1 of the 7 alleles used to determine sequence type (Feil *et al.*, 2003). Although *S. aureus* strain lineage and gene prevalence does not correlate with disease causation in humans (Feil *et al.*, 2003, Lindsay *et al.*, 2006), *S. aureus* strains of clonal lineages CC5 and CC30 have been associated with the development of haematogenous complications of *S. aureus* bacteraemia, including infective endocarditis (Fowler *et al.*, 2007).

1.3.4 *S. aureus* virulence factors implicated in the pathogenesis of infective endocarditis

Virulence factors are components of an organism that contribute to disease pathogenesis, and are often identified in animal models of infection (Foster, 2005). *S. aureus* produces a great array of virulence factors, including secreted toxins, enzymes and proteins, and anchorless and cell wall-anchored proteins, as outlined in Table S1.1. A selection of *S. aureus* virulence factors, including those implicated in the pathogenesis of infective endocarditis, are discussed below in further detail.

1.3.4.1 *S. aureus* toxins

Toxins produced by *S. aureus* include α-, β-, δ- and γ-toxins, which induce lysis of host cells as described in Table S1.1 (Dinges *et al.*, 2000, Lowy, 1998). α-toxin has been implicated in the destruction of valvular tissue following *S. aureus* phagocytosis by endothelial cells (see Section 1.4.3.2) (Chorianopoulos *et al.*, 2009, Sinha & Herrmann, 2005). Although α-toxin has been reported to induce aggregation of human platelets *in vitro* (Bhakdi *et al.*, 1988), a strain expressing increased levels of α-toxin had paradoxically reduced virulence in a rabbit model of infective endocarditis, possibly due to α-toxin-mediated platelet lysis and release of platelet microbicidal proteins (PMPs) (Bayer *et al.*, 1997) (see Section 1.5.2). α-toxin has also been demonstrated to repress regional cardiac perfusion and myocardial contractility in rats via increased generation of thromboxane and eicosanoid release (Grandel *et al.*, 2009).

Other toxins include *S. aureus* superantigens, of which at least 18 have been identified to date, and consist of enterotoxins and toxic shock syndrome toxin-1 (TSST-1), responsible for staphylococcal food poisoning and toxic shock syndrome, respectively, as outlined in Table S1.1 (Fraser & Proft, 2008). Superantigens are so named because they are able to induce T-cell proliferation and cytokine release by binding to major histocompatibility class (MHC) II complexes on T-cells (Fraser &

Proft, 2008). Exfoliative toxins such as epidermolytic toxins A and B are responsible for desquamating skin conditions such as staphylococcal scalded skin syndrome (Dinges *et al.*, 2000). In addition, the leucocytolytic toxin PVL has been implicated in severe cutaneous infection as detailed in Table S1.1 (Foster, 2005).

1.3.4.2 *S. aureus* enzymes

S. aureus also produces enzymes, including proteases, lipases, hydrolases and collagenase, which contribute to nutrient acquisition and host tissue destruction, facilitating survival in the host (Table S1.1) (Dinges *et al.*, 2000, Lowy, 1998, Novick, 2003). β-lactamase mediates *S. aureus* resistance to β-lactam antibiotics (Lowy, 1998). Coagulase and staphylokinase have been implicated in clot formation and dissolution respectively, however neither influences the development of infective endocarditis (Table S1.1) (Baddour *et al.*, 1992, Bokarewa *et al.*, 2006, Lowy, 1998, Moreillon *et al.*, 1995). Specifically, Ruotsalainen *et al* did not identify any difference in the prevalence of genes encoding staphylokinase between *S. aureus* strains isolated from cases of bacteraemia and infective endocarditis (Ruotsalainen *et al.*, 2008). However, staphylokinase may facilitate dissolution and fragmentation of vegetations in infective endocarditis resulting in embolism, although this has not been examined to date (Bokarewa et al., 2006). In addition, the presence of coagulase did not alter early vegetation formation in a rat model of infective endocarditis, but its role in the later stages of infective endocarditis or in the disease process in humans is unknown (Moreillon *et al.*, 1995).

1.3.4.3 Other *S. aureus* secreted proteins, cell wall and capsule

S. aureus secretes a number of proteins, including chemotaxis inhibitory protein of staphylococci (CHIPS), staphylococcal complement inhibitor (SCIN), extracellular adhesion protein (Eap) and staphylococcal superantigen-like proteins (SSLs), with roles of these virulence factors outlined in Table S1.1 (Foster, 2005, Fraser & Proft,

2008, Sinha & Herrmann, 2005). SSLs are so named because they share sequence similarity to superantigens such as TSST-1 (Fraser & Proft, 2008). Fourteen SSLs have been identified to date but rather than bind MHC II complexes, they bind IgA, complement components and inhibit neutrophil binding to endothelium, inhibiting host immune activity (Fraser & Proft, 2008). SSL-5 is also able to induce platelet aggregation via a platelet glycoprotein (GP)Ib receptor-dependent mechanism, but its role in the induction of infective endocarditis in animal models has not been examined to date (de Haas *et al.*, 2009).

The *S. aureus* cell wall, mainly consisting of peptidoglycan, mediates platelet aggregation at the stationary phase of growth (Kessler *et al.*, 1991), while lipoteichoic acid, which forms part of the cell wall, independently inhibits platelet aggregation induced by pharmacological agonists (Sheu *et al.*, 2000). The role of the *S. aureus* cell wall in the induction of infective endocarditis is unknown. The outer *S. aureus* capsule inhibits bacterium-platelet interactions (see Section 1.3.5.2) and prevents complement recognition by leucocytes, thus reducing phagocytosis (Foster, 2005, Risley *et al.*, 2007).

1.3.4.4 Cell wall-associated proteins of *S. aureus*

A number of anchorless and cell wall-anchored adhesins are expressed on the surface of *S. aureus*, and described in Table S1.1. Anchorless proteins, also termed secretable expanded repertoire adhesive molecules (SERAMs), are bound non-covalently to the cell wall upon secretion, and include the extracellular matrix (ECM)-binding protein homologue and the ECM protein-binding protein (Emp) that mediate adherence to plasma proteins and endothelial cells, as well as contributing to biofilm formation (Johnson *et al.*, 2008, Sinha & Herrmann, 2005). Extracellular fibrinogen-binding protein (Efb) is multifunctional as it interferes with immune defence, wound healing and platelet aggregation (Shannon & Flock, 2004). Other anchorless proteins include autolysins, while PBPs are attached to the cytoplasmic membrane, and elastin-binding protein is transmembranous and enables *S. aureus* to

bind elastin (Clarke & Foster, 2006, Downer *et al.*, 2002, Lowy, 1998). The contribution of *S. aureus* anchorless proteins to the pathogenesis of infective endocarditis is not known.

A large number of cell wall-anchored proteins such as plasmin-sensitive protein (Pls), serine-rich adhesin for platelets (SraP) and Microbial Surface Components Recognising Adhesive Matrix Molecules (MSCRAMMs) are expressed by *S. aureus* (Table S1.1) (Foster & Hook, 1998, Juuti *et al.*, 2004, Siboo *et al.*, 2005). The gene encoding Pls is only present in type I SCC*mec* found in MRSA strains (Juuti *et al.*, 2004). Pls may contribute to nasal colonisation by binding nasal epithelium, but is associated with inhibition of platelet aggregation, reduced fibronectin binding and cellular invasion, possibly via decreased expression of clumping factor A (ClfA) and steric hindrance of fibronectin-binding proteins (FnBPs), respectively (Hussain *et al.*, 2009, Juuti *et al.*, 2004). Conversely, SraP binds platelets and is associated with increased virulence in a rabbit model of infective endocarditis (Siboo *et al.*, 2005).

1.3.4.4.1 *S. aureus* MSCRAMMs

The term MSCRAMM has been coined to describe the cell wall-anchored proteins present on *S. aureus* that bind ECM proteins (Foster & Hook, 1998). Twenty-one MSCRAMMs have been identified to date and include fibronectin-binding proteins A and B (FnBPA, FnBPB), clumping factors A and B (ClfA, ClfB), serine-aspartate repeat (Sdr) proteins, staphylococcal protein A (SpA), collagen adhesin (Cna), *S. aureus* surface (Sas) proteins and iron-regulated surface determinants A and B (IsdA, IsdB) (Table S1.1) (Clarke & Foster, 2006, Roche *et al.*, 2003).

The main roles of MSCRAMMs are to facilitate bacterial adhesion to ECM, evasion of host immune defences and internalisation by host cells, culminating in disease pathogenesis (Clarke & Foster, 2006). FnBPs, SpA, Clf and Isd proteins are discussed in further detail (see Section 1.3.5). Cna contributes to *S. aureus* adherence to valvular sub-endothelium (see Section 1.4.3.2), but is predominantly involved in

the development of bone and joint infections (Clarke & Foster, 2006). SdrC and SdrD have been implicated in nasal colonisation, while SdrE weakly induces platelet aggregation (Corrigan *et al.*, 2009, O'Brien *et al.*, 2002a). SasC facilitates biofilm formation, SasG has been implicated in biofilm formation, nasal colonisation and inhibition of the ligand-binding activities of other MSCRAMMs, and SasH has been associated with the development of invasive *S. aureus* infection (Clarke & Foster, 2006, Roche *et al.*, 2003). However, the role of *S. aureus* Sas and Sdr proteins in the pathogenesis of infective endocarditis is unknown.

Many MSCRAMMs bind multiple ligands, yet there is also redundancy as a number of MSCRAMMs are capable of binding the same ligand (Clarke & Foster, 2006). For example, FnBPA adheres to both fibrinogen and fibronectin at the mid-exponential phase of growth, while ClfA adheres to fibrinogen, predominantly at the stationary phase of growth, and both have been implicated in the pathogenesis of infective endocarditis (see Section 1.3.5) (Clarke & Foster, 2006). This plasticity in ligand-binding ability appears to be beneficial for *S. aureus*, as it facilitates host colonisation and disease causation under varying environmental conditions and during different growth phases, accounting for the capacity of this pathogen to infect a range of hosts and cause invasive and non-invasive diseases (Table 1.3) (Clarke & Foster, 2006).

1.3.4.4.2 Structure of *S. aureus* MSCRAMMs

The structure of selected MSCRAMMs, outlining binding regions and ligands are depicted in Figure 1.1. The N-terminus contains the approximately 40 amino acid long signal sequence (S), which facilitates secretion of the MSCRAMM through the cell membrane. Adjacent to this are the A to E or N-terminal NEAr Transporter (NEAT) ligand-binding domains, binding fibrinogen, fibronectin, von Willebrand factor (vWF), complement, IgG, elastin, cytokeratin, haem and transferrin as outlined (Clarke & Foster, 2006, Clarke *et al.*, 2004, Mazmanian *et al.*, 2003). The serine-aspartate dipeptide repeats (R) facilitate extension of the ligand-binding regions (Hartford *et al.*, 1997). The wall-spanning region (W) is composed of

proline and glycine residues and includes octapeptide (Wr) and non-repeat (Wc) regions, while the hydrophobic membrane-spanning region (M) at the C-terminus contains positively-charged residues (Clarke & Foster, 2006).

The LPXTG amino acid motif is located between regions W and M and plays a crucial role in the attachment of MSCRAMMs to the cell wall peptidoglycan (Clarke & Foster, 2006). Cleavage of LPXTG between the threonine and glycine residues by the membrane bound transpeptidase Sortase A, establishes an amide bond between the carboxyl group of threonine and the amino group of the cell wall, anchoring the MSCRAMM (Clarke & Foster, 2006).

MSCRAMMs can either have a circumferential or punctate distribution on the *S. aureus* cell surface, with a circumferential distribution conferred by the presence of a YSIRK/GS amino acid motif 18 to 20 residues upstream of the peptide cleavage site in MSCRAMMs such as ClfA, FnBPB and SpA (DeDent *et al.*, 2008).

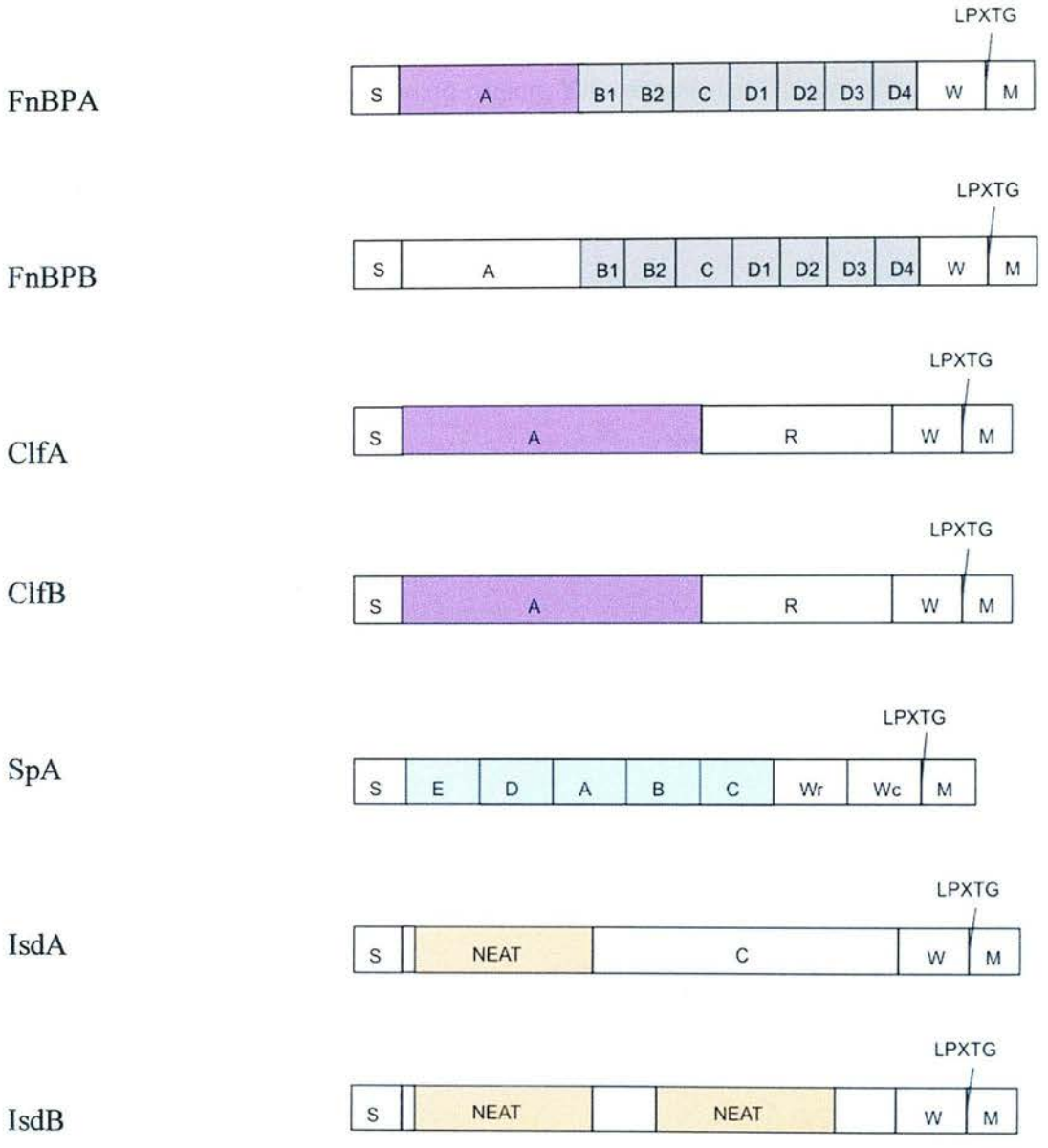

Figure 1.1. The structural organisation of *S. aureus* Microbial Surface Components Recognising Adhesive Matrix Molecules (MSCRAMMs). Regions in purple bind fibrinogen, in grey bind fibronectin and in blue bind von Willebrand factor, complement and IgG-Fc fragments. Additionally, the A domain of FnBPA binds elastin and the A domain of ClfB binds cytokeratin. N-terminal NEAr Transporter (NEAT) domains are able to bind haem and transferrin and in the case of IsdA, also bind fibrinogen, fibronectin and cytokeratin. Regions are not drawn to scale. FnBPA, fibronectin-binding protein A; FnBPB, fibronectin-binding protein B; ClfA, clumping factor A; ClfB, clumping factor B; SpA, staphylococcal protein A; IsdA, iron-regulated surface determinant A; IsdB, iron-regulated surface determinant

B. S, signal sequence; letters A-E, ligand-binding domains; R, serine-aspartate dipeptide repeats; W, wall-spanning region; Wr, octapeptide repeat; Wc, non-repeat region; M, membrane-spanning region; LPXTG, the amino acid motif that is cleaved by Sortase A. Adapted from references (Clarke & Foster, 2006, Foster & Hook, 1998).

1.3.5 The role of MSCRAMMs in the pathogenesis of *S. aureus* infective endocarditis

1.3.5.1 Fibronectin-binding proteins (FnBPs)

FnBPA and FnBPB are encoded by genes *fnbA* and *fnbB*, respectively (Greene *et al.*, 1995). Strain-dependent variation in the distribution of *fnb* genes has been observed, with 77% of *S. aureus* clinical isolates possessing both genes and 23% carrying one gene (Peacock *et al.*, 2000). Although isolates associated with invasive disease are more likely to contain both *fnb* genes, this does not correlate with the ability of these strains to bind immobilised fibronectin *in vitro* (Peacock *et al.*, 2000). Maximal expression of both FnBPs occurs during the mid-exponential phase of growth, with positive regulation by staphylococcal accessory regulator A (SarA), sigma B (σ^B) and *S. aureus* exoprotein expression *(sae)* and negative control by *agr* (See Section 1.3.6) (Cheung *et al.*, 2009, Entenza *et al.*, 2005, Wolz *et al.*, 2000).

The FnBPA A region binds elastin and the γ chain of fibrinogen (Figure 1.1), whereas the BCD regions of both FnBPs bind the F1 region of the N-terminal domain of fibronectin using a β-zipper mechanism (Clarke & Foster, 2006, Keane *et al.*, 2007). Eleven fibronectin-binding repeat regions have been identified within the BCD domains of both FnBPA and FnBPB, with six repeats exhibiting evidence of multivalent binding in each MSCRAMM (Meenan *et al.*, 2007).

Using a genetically diverse collection of *S. aureus* strains, Loughman *et al* detected 24% to 34% amino acid sequence diversity in the A region of FnBPs with 7 different alleles identified for FnBPA and 3 different isotypes for FnBPB (Loughman *et al.*, 2008). Variation in the amino acid sequence of the A domain was confined to the N2N3 regions with minimal involvement of the ligand-binding and latch-forming regions, thus conferring antigenic variation with preserved fibrinogen- and elastin-binding affinity and ability to cause disease (Loughman *et al.*, 2008). Sequence diversity of the A domain of FnBPs does not correlate with *S. aureus* clonal origin

(ST), suggesting that this allelic variation developed via horizontal gene transfer or recombination (Loughman *et al.*, 2008).

FnBPA and FnBPB share 95% amino acid identity in the D, W and M regions, suggesting that they arose as a result of gene duplication (Sinha and Herrmann, 2005). However, the A regions of FnBPA and FnBPB have only 45% amino acid identity, and this is reflected in their varying ability to bind plasma proteins as the A domain of FnBPA binds fibrinogen and elastin, while the FnBPB A domain does not exhibit any ligand-binding ability (Clarke & Foster, 2006, Keane *et al.*, 2007). In spite of the fact that both the ClfA and FnBPA A domains bind fibrinogen at its γ chain, they only share 25% to 29% amino acid identity (Clarke and Foster, 2006).

Early animal experiments provided conflicting evidence as to the role of FnBPA in the pathogenesis of infective endocarditis. Kuypers *et al* discovered that vegetations infected with low fibronectin-binding mutants of *S. aureus* had 100-fold reduced bacterial densities (Kuypers & Proctor, 1989), while *S. aureus* mutants deficient in *sar* alone or both *sar* and *agr* exhibited reduced attachment to vegetations, attributed to the loss of fibronectin-binding capacity (Cheung *et al.*, 1994). Furthermore, the double *sar/agr* mutant was reported to have an attenuated ability to persist and multiply within vegetations (Cheung *et al.*, 1994). However, other studies have not identified differences in vegetation bacterial counts with *sar* and *agr* single and double mutants or with strains deficient in FnBPs, despite variation in fibronectin binding *in vitro* (Flock *et al.*, 1996, Xiong *et al.*, 2004).

MSCRAMM redundancy may have been responsible for these inconclusive results. FnBPA, FnBPB and ClfB are all expressed during the mid-exponential phase of growth, and FnBPA-deficient mutants may therefore retain ligand-binding activity and exhibit no difference in their ability to induce experimental infective endocarditis (Que *et al.*, 2001). In order to address this issue, MSCRAMMs were expressed in a heterologous host, *Lactococcus lactis* (Que *et al.*, 2001). Significantly smaller inocula of FnBPA- or ClfA-expressing *L. lactis* strains, were required to

induce endocarditis as compared to the parent *L. lactis* strain, suggesting a role for these MSCRAMMs in the pathogenesis of infective endocarditis (Que *et al.*, 2001).

Further studies were performed by the same group to determine the precise role and interplay of FnBPA and ClfA in the pathogenesis of infective endocarditis. In catheter-induced models of infective endocarditis, both ClfA- and FnBPA-expressing *L. lactis* were present within cardiac vegetations, but ClfA-positive strains were confined to vegetations and eradicated within 10 d, while FnBPA-positive strains persisted with evidence of invasion of adjacent endothelium (Piroth *et al.*, 2008, Que *et al.*, 2005). Loss of fibrinogen-binding capacity completely abrogated colonisation and reduced fibronectin-mediated cell internalisation in these studies, suggesting a synergistic role for these MSCRAMMs in the pathogenesis of infective endocarditis (Piroth *et al.*, 2008, Que *et al.*, 2005).

Piroth *et al* have recently demonstrated that the $A4^{+16}$ domain of FnBPA is crucial for both fibrinogen and fibronectin binding, and when expressed on *L. lactis*, independently mediates development of infective endocarditis in an experimental rat model (Piroth *et al.*, 2008). Pathogenesis of infective endocarditis correlated with fibrinogen-binding capacity, while disease severity correlated with both fibrinogen and fibronectin binding, suggesting synergistic roles for these ligand-binding activities in infective endocarditis (Piroth *et al.*, 2008).

In a study analysing the capacity of both FnBPA and FnBPB to induce platelet aggregation following expression in *Staphylococcus carnosus*, FnBPA, but not FnBPB, was able to induce fibrinogen- and fibronectin-mediated platelet aggregation *in vitro* (Heilmann *et al.*, 2004). However, a more recent study has demonstrated that both FnBPs are capable of inducing platelet aggregation *in vitro* (Fitzgerald *et al.*, 2006b). The role of FnBPB in the induction of infective endocarditis in experimental animal models has not been investigated.

FnBPs also facilitate *S. aureus* invasion of a wide variety of host cells, including endothelial cells, epithelial cells, keratinocytes, osteoblasts and alveolar cells, and

FnBP-mediated endothelial invasion contributes to the pathogenesis of infective endocarditis (see Section 1.4.3.2) (Clarke & Foster, 2006, McElroy *et al.*, 2002). FnBPs facilitate biofilm development by MRSA strains (O'Neill *et al.*, 2008, Vergara-Irigaray *et al.*, 2009), but are paradoxically associated with reduced virulence in animal models of pneumonia, possibly via FnBPA-mediated phagocytosis and elimination of *S. aureus* from murine lungs (McElroy *et al.*, 2002).

1.3.5.2 Clumping factor A (ClfA)

ClfA and ClfB are members of the Sdr family of MSCRAMMs, characterised by serine-aspartate dipeptide repeats within the R region (Figure 1.1) (Clarke & Foster, 2006). The A domain of Clf proteins is approximately 520 amino acids long in most strain, and at the N2 and N3 domains binds the C-terminal of the fibrinogen γ chain at residues H6, H7, G10, Q13, A14 and G15 (Ganesh *et al.*, 2008, McDevitt *et al.*, 1997). Clumping factor-mediated fibrinogen binding occurs via a 'latch and dock mechanism' by the formation of an IgG-like fold between the N2 and N3 domains, similar to the mode of fibrinogen binding employed by *Staphylococcus epidermidis* SdrG (Foster & Hook, 1998, Ganesh *et al.*, 2008). ClfA binds fibrinogen at the same region as the platelet glycoprotein (GP)IIb/IIIa receptor, and this interaction is inhibited by calcium binding to the EF-hand motif of the A region (Ganesh *et al.*, 2008, McDevitt *et al.*, 1997).

The R region of ClfA, which is 308 amino acids long in *S. aureus* Newman, does not possess any intrinsic ligand-binding activity, but facilitates extension of the ligand-binding A region (Hartford *et al.*, 1997). Unlike the A domain, region R has been reported to vary in size from 193 to 440 amino acids between different strains, but this does not influence fibrinogen binding (McDevitt & Foster, 1995). ClfA is encoded by the *clfA* gene found in all strains of *S. aureus* (Tristan *et al.*, 2003), and is predominantly expressed during the stationary phase of growth *in vitro* and *in vivo* under the control of σ^B but independent of *agr* (see Section 1.3.6) (Entenza *et al.*, 2005, Goerke *et al.*, 2005, Wolz *et al.*, 1996).

Studies have demonstrated that ClfA contributes to the early stages of development of experimental infective endocarditis through its ability to bind fibrinogen (Entenza *et al.*, 2005, Moreillon *et al.*, 1995, Sullam *et al.*, 1996). To avoid the possibility of MSCRAMM functional overlap, Stutzmann Meier *et al* used *Streptococcus gordonii* as a heterologous host for ClfA expression in a rat model of infective endocarditis (Stutzmann Meier *et al.*, 2001). ClfA-positive strains were associated with significantly increased vegetation adherence with no effect on vegetation bacterial densities (Stutzmann Meier *et al.*, 2001), confirming the primary role of ClfA in vegetation colonisation (Que *et al.*, 2005). However, the importance of ClfA in the pathogenesis of infective endocarditis has recently been questioned as ClfA-induced fibrinogen binding and induction of infective endocarditis in animal models is inhibited by *S. aureus* capsule, which is present *in vivo*, but often absent when cells are processed for the above experiments (Baddour *et al.*, 1992, Risley *et al.*, 2007).

It has also been postulated that ClfA promotes the development of arthritis and inhibits phagocytosis by fibrinogen-dependent and -independent mechanisms, the latter involving enhanced inactivation of complement C3b by the ClfA A domain binding serum factor I (Clarke & Foster, 2006, Hair *et al.*, 2008, Higgins *et al.*, 2006).

1.3.5.3 Clumping factor B (ClfB)

In contrast to the FnBPs which contain almost identical D regions, there is only 27% amino acid identity between ClfA and ClfB, suggesting that they arose independently of each other (Clarke & Foster, 2006). This is manifest in variation in their ligand-binding ability, as instead of binding the γ chain of fibrinogen, the A domain of ClfB binds repeat region 5 within the C-terminal end of the Aα chain of fibrinogen (Ni Eidhin *et al.*, 1998, Walsh *et al.*, 2008). Furthermore, ClfB binds fibrinogen less efficiently than ClfA, as each molecule of fibrinogen adheres to two ClfA molecules or one ClfB protein (Clarke & Foster, 2006, Miajlovic *et al.*, 2007).

ClfB binds avidly to cytokeratin-10 on the surface of squamous epithelial cells, facilitating persistent nasal colonisation in both animals and humans (O'Brien *et al.*, 2002b, Walsh *et al.*, 2008, Wertheim *et al.*, 2008). In nutrient-rich media, ClfB is only expressed during the exponential phase of growth, as it is later cleaved between the N1 and N2 domains by *S. aureus* aureolysin (Ni Eidhin *et al.*, 1998, Walsh *et al.*, 2008). However, intact ClfB is present late into the stationary phase of growth when cultured in nutrient-deplete media (Wertheim *et al.*, 2008).

As ClfA and ClfB are maximally expressed at different growth phases, it has been postulated that they work synergistically in the development of infective endocarditis. The single and double ClfA- and ClfB-negative derivatives of *S. aureus* strain Newman, which lacks functional FnBPs (Grundmeier *et al.*, 2004), had longer times to platelet aggregation than the wild type *in vitro* (Loughman *et al.*, 2005, Miajlovic *et al.*, 2007, O'Brien *et al.*, 2002a). In addition, *L. lactis* expressing ClfA had a shorter lag time to platelet aggregation than ClfB-expressing strains, suggesting that ClfA is more potent than ClfB in inducing platelet aggregation (O'Brien *et al.*, 2002a). Conversely, neither single nor double ClfA- and ClfB-mutants affected the ability of *S. aureus* to infect cardiac vegetations in a catheter-induced rat model of infective endocarditis (Entenza *et al.*, 2000).

1.3.5.4 Staphylococcal protein A (SpA)

SpA is a 42 kDa protein that comprises 7% of the *S. aureus* cell wall and is expressed in most isolates (Gao & Stewart, 2004, Nguyen *et al.*, 2000). It was the first cell wall-associated protein to be identified, yet its structure is different from most other MSCRAMMs (Fig. 1.1) (Clarke & Foster, 2006). SpA contains four or five 60 amino acid long ligand-binding domains (D, E, A, B and C), forming three α helices at its N-terminus (Sjodahl, 1977). Expression is positively regulated by SarS, SarT and repressor of toxins (*rot*) and negatively regulated by SarA either alone or in combination with *agr* (see Section 1.3.6) (Cheung *et al.*, 1997, Gao & Stewart, 2004). Maximal expression of SpA occurs during the exponential phase of

growth in most strains (Gao & Stewart, 2004).

SpA confers a survival advantage to *S. aureus* by phagocytosis avoidance, as it binds the Fc portion of IgG, altering its orientation and preventing the antibody from binding leucocytes (Clarke & Foster, 2006). SpA also binds the $F(ab')_2$ region of certain antibodies, IgM, vWF, complement and tumour necrosis factor receptor-1 (Clarke & Foster, 2006, Nguyen *et al.*, 2000), and contributes to the development of abscesses in murine infection models (Cheng *et al.*, 2009).

Although early research suggested that cell wall SpA bound to IgG-Fc was required for platelet aggregation (Hawiger *et al.*, 1979), a more recent study has demonstrated that SpA is not capable of independent platelet aggregation and instead contributes to ClfA-mediated thrombus formation (O'Brien *et al.*, 2002a). SpA-mediated *S. aureus*-platelet binding is thought to be particularly important under the high shear conditions present *in vivo* (see Section 1.4.4.3).

1.3.5.5 Iron-regulated surface determinants (Isd proteins)

Although the above studies have identified putative MSCRAMMs implicated in the pathogenesis of infective endocarditis, the employed growth conditions do not accurately reflect the *in vivo* milieu, particularly as the artificial growth media utilised for bacterial culturing are nutrient-rich. In contrast, human blood only contains 10^{-18} M free iron as iron is insoluble in the presence of oxygen (Pluym *et al.*, 2008). Most iron within the bloodstream is stored within binding proteins such as haemoglobin, transferrin and lactoferrin (Ahn *et al.*, 2004, Ward *et al.*, 1996). This iron-deplete environment appears to form part of the immune response to microbial infection as bacteria, like other organisms, require iron for oxidative and metabolic processes and hence survival (Ahn *et al.*, 2004, Skaar *et al.*, 2004). In addition, iron deficiency is thought to mediate the bacteriocidal and bacteriostatic effects of plasma proteins by inhibition of bacterial RNA synthesis (Ward *et al.*, 1996).

Consequently, organisms such as *S. aureus* have adapted to the hostile *in vivo* environment by the production of iron-uptake systems such as siderophores to remove transferrin-bound iron, and specific receptors to remove iron from the tetrapyrrole haem (Skaar *et al.*, 2004). Haem iron, present in haemoglobin and myoglobin, is the favoured iron source for *S. aureus*, particularly during the mid-exponential phase of growth, with increased uptake of iron from transferrin in later growth phases (Skaar *et al.*, 2004, Torres *et al.*, 2006).

Haem iron acquisition by *S. aureus* is facilitated by the Isd pathway, consisting of IsdA to IsdI (Mazmanian *et al.*, 2003). A model for the Isd system involves the actions of α-toxin on erythrocytes, liberating haemoglobin, which binds IsdB, and to a lesser degree IsdH, and is converted to haem (Pishchany *et al.*, 2009, Torres *et al.*, 2006). Haem is then transferred to IsdA, transported to cell wall IsdC and taken up by adenosine triphosphate (ATP)-binding cassette transporters IsdD to IsdF (Liu *et al.*, 2008, Mazmanian *et al.*, 2003). Iron is removed from haem by haem oxygenases IsdG and IsdI within the cytoplasm and utilised for metabolic processes (Mazmanian *et al.*, 2003).

The *isd* locus is present in all *S. aureus* genomes, with 8 genes encoding 3 iron-regulated transcriptional units (Vermeiren *et al.*, 2006). Transcription of Isd proteins is increased in low environmental iron conditions, maintained by negative feedback from the ferric uptake repressor (*fur*) (Clarke *et al.*, 2004). IsdA and IsdB are expressed on the cell wall and along with IsdH contain LPXTG motifs, while IsdC contains an NPQTN sorting signal and is attached to the cell wall envelope by Sortase B (Mazmanian *et al.*, 2002). Each Isd protein contains at least one copy of the 150 amino acid long NEAT domain, which is required for haem binding (Figure 1.1) (Andrade *et al.*, 2002).

1.3.5.5.1 Iron-regulated surface determinant A (IsdA)

Whereas expression of other MSCRAMMs is downregulated during growth in body

fluids, IsdA is preferentially expressed during growth in human serum, dialysate and other low iron conditions (Clarke *et al.*, 2004, Morrissey *et al.*, 2002, Wiltshire & Foster, 2001). IsdA binds fibronectin as well as both the Bβ and γ chains of fibrinogen at its NEAT domain, with twenty times the affinity of ClfA for fibrinogen (Clarke *et al.*, 2004). IsdA, particularly in combination with ClfB or SdrCDE, binds cytokeratin-10 and locirin in desquamated human nasal epithelial cells at its NEAT domain, mediating nasal colonisation (Clarke *et al.*, 2009, Corrigan *et al.*, 2009). It is also able to bind asialofetuin, fetuin, haem, haemoglobin and transferrin (Clarke *et al.*, 2004, Vermeiren *et al.*, 2006). Expression of IsdA is maximal at the early stationary phase of growth (Clarke *et al.*, 2004, Corrigan *et al.*, 2009).

The C domain of IsdA plays a role in mediating resistance to host defences such as sebum, C-6-H, lactoferrin and antibiotics and facilitates survival on human skin (Clarke & Foster, 2008, Clarke *et al.*, 2007). IsdA is associated with reduced biofilm formation in some strain backgrounds, but not others (Clarke *et al.*, 2007). The role of IsdA in platelet adhesion, aggregation and the pathogenesis of infective endocarditis has not been investigated to date.

1.3.5.5.2 Iron-regulated surface determinant B (IsdB)

IsdB is a cell wall-anchored MSCRAMM which contains two NEAT domains (Figure 1.1) and has a 19-fold greater expression in low, as opposed to high, iron conditions (Mazmanian *et al.*, 2002, Torres *et al.*, 2006, Pishchany *et al.*, 2009). It co-localises with IsdA on the *S. aureus* cell surface, forming a complex at the site of cell division (Pishchany *et al.*, 2009). With decreasing iron concentrations, IsdA and IsdB display a circumferential distribution on the cell wall, while a more punctate and discrete distribution is observed at high iron concentrations (Pishchany *et al.*, 2009).

Both IsdA and IsdB are expressed on the *S. aureus* cell surface in murine infection

models, and have been detected with increased expression in the heart (Pishchany *et al.*, 2009, Torres *et al.*, 2006). Furthermore, *S. aureus* strains lacking IsdB are associated with reduced virulence in mouse models of abscess formation and bacteraemia (Cheng *et al.*, 2009, Pishchany *et al.*, 2009, Torres *et al.*, 2006). However it is not known whether IsdB can bind plasma proteins, induce platelet aggregation or contribute to the pathogenesis of infective endocarditis.

1.3.6 The genetic regulation of *S. aureus* MSCRAMM expression

Expression of MSCRAMMs is under tight genetic regulation. There are a multitude of regulatory systems, including *agr, sae,* staphylococcal respiratory response (*srr*), autolysis-related locus (*arl*), *sar, rot,* σ^B and *fur*, that control the expression of *S. aureus* virulence factors (Fournier & Hooper, 2000, Harraghy *et al.*, 2005, Novick, 2003, Yarwood *et al.*, 2001).

Agr plays an important role in the regulation of *S. aureus* virulence (Novick, 2003). It is one of many two-component systems, so named because they consist of a sensor histidine kinase and a response regulator, which enable gene expression to be modulated by environmental stimuli (Blevins *et al.*, 2002, Novick, 2003). The main effects of *agr* are to upregulate exoprotein and downregulate surface protein expression during the post-exponential phase of growth (Blevins *et al.*, 2002, Novick, 2003, Wolz *et al.*, 1996, Xiong *et al.*, 2004). *Agr* has also been reported to have important metabolic effects (Dunman *et al.*, 2001).

Agr is stimulated by an extracellular ligand known as the auto-inducing peptide, derived from AgrD (Figure 1.2) (Novick, 2003). This binds to the signal receptor AgrC, leading to activation of the two-component system and upregulation of promoters P2 and P3 through AgrA (Novick, 2003). The resultant expression of RNAIII leads to reduced synthesis of some MSCRAMMs, increased production of exoproteins and overall increased bacterial virulence (Blevins *et al.*, 2002, Cheung *et al.*, 1994).

The *S. aureus* genome encodes at least 15 other two-component systems (Cheung *et al.*, 2004). One example is *sae*, which is transcribed at the early exponential phase of growth, and in the presence of *agr*, is expressed during the post-exponential growth phase. *Sae* controls nuclease and coagulase production and also upregulates FnBP expression (Cheung *et al.*, 2009, Novick, 2003). *ArlRS* is another two-component system that appears to be upregulated by *agr*, but is itself able to downregulate *agr*, leading to a reduction in exoprotein synthesis (Novick, 2003). *SrrAB* facilitates *S. aureus* growth in anaerobic conditions and is mutually downregulatory with *agr* (Novick, 2003).

Sar encodes three overlapping transcripts – SarA, SarB and SarC, transcribed from promoters P1, P2 and P3 respectively (Cheung *et al.*, 1992, Wolz *et al.*, 2000). SarA and SarB are upregulated during the exponential phase of growth, whilst SarC expression is upregulated in the post-exponential growth phase by the σ^B-dependent P3 promoter (Blevins *et al.*, 2002, Novick, 2003, Wolz *et al.*, 2000). SarA is the most studied component and is known to bind AT-rich sequences within numerous genes, including those between the *agr* promoters P2 and P3, facilitating RNAIII production (Figure 1.2) (Novick, 2003). *Sar* has therefore been described as a transcription factor (Novick, 2003). SarA upregulates MSCRAMM expression and downregulates exoprotein and SpA synthesis during the exponential phase of growth (Blevins *et al.*, 2002, Novick, 2003). There is ongoing debate as to whether *sar* or *agr* is more important in the regulation of MSCRAMM expression (Cheung *et al.*, 1992, Novick, 2003).

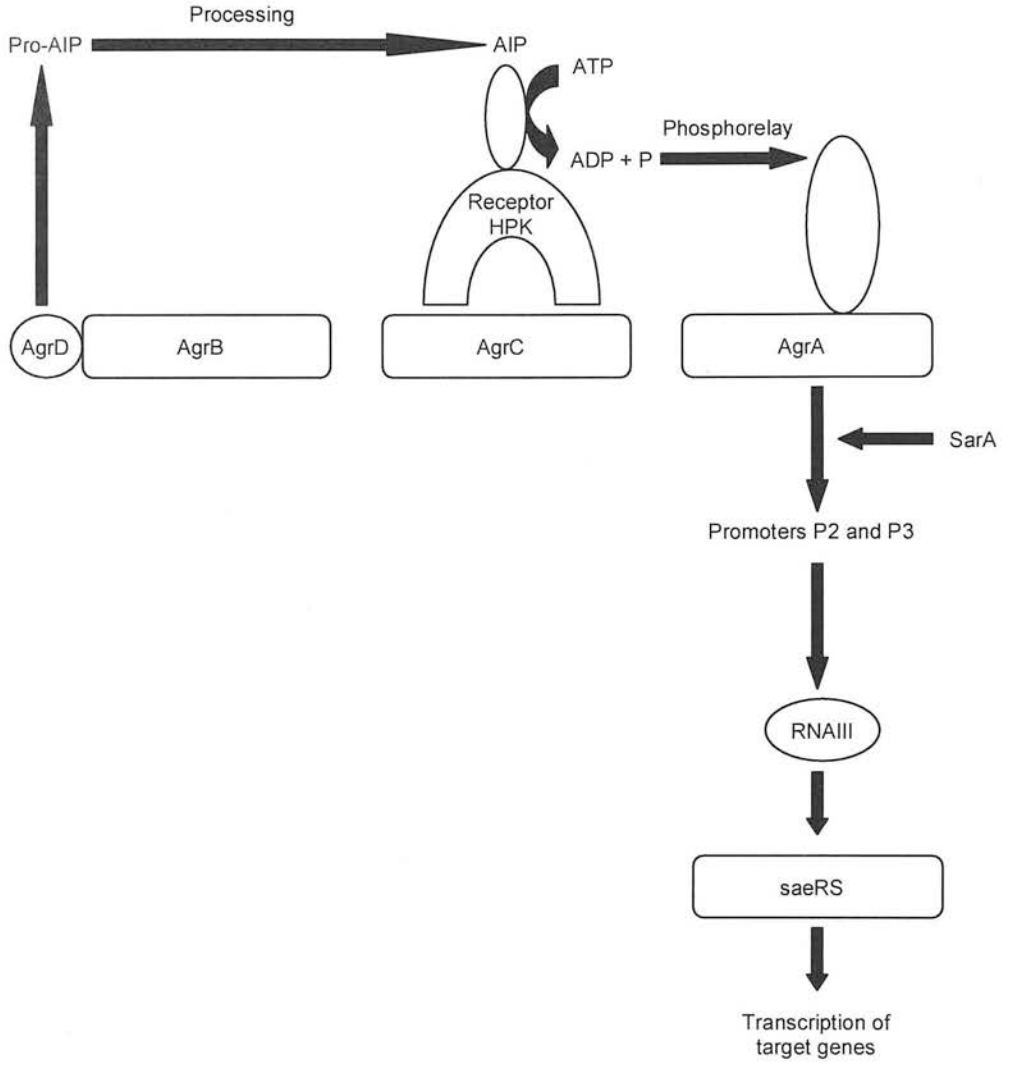

Figure 1.2. The accessory gene regulator (*agr*) system. The auto-inducing pro-peptide (pro-AIP), derived from AgrD, is processed and secreted by AgrB, binds to an extracellular histidine protein kinase (HPK) loop in the AgrC receptor, leading to auto- or dephosphorylation in AgrC and AgrA. AgrA, in conjunction with staphylococcal accessory regulator A (SarA,) activates the two *agr* promoters P2 and P3, resulting in the production of RNAIII, which via other two-component systems such as *S. aureus* exoprotein expression (sae)RS, controls transcription of target genes. Adapted from (Novick, 2003).

σ^B and *rot* have been described as virulence modulators (Entenza *et al.*, 2005, Gao & Stewart, 2004, Novick, 2003). *Rot* is part of the *sar* family and has been reported to positively regulate SarS and thus SpA expression indirectly (Said-Salim *et al.*, 2003). σ^B is an alternative sigma factor which, rather than being activated through two-component systems, is directly activated within the cell by environmental stimuli (Novick, 2003). It upregulates MSCRAMM expression, but unlike SarA, mainly has its effects during the post-exponential phase of growth (Entenza *et al.*, 2005, Novick, 2003). It is feasible that there are *S. aureus* gene regulators that are still to be identified (van Belkum *et al.*, 2009).

1.4 Cardiac vegetations

1.4.1 The role of cardiac vegetations in infective endocarditis

Vegetations are bacterial-platelet-fibrin complexes which form on native and prosthetic cardiac valves and implanted pacemaker leads in infective endocarditis (Durack, 1975). They are identified in 50% to 80% of patients with infective endocarditis using transthoracic echocardiography and 75% to 95% of patients using transoesophageal echocardiography (De Castro *et al.*, 1997, Di Salvo *et al.*, 2001, Thuny *et al.*, 2005, Vilacosta *et al.*, 2002). Vegetations contribute to bacterial persistence by facilitating evasion of the host immune system, antibiotic therapies and the shear forces of arterial blood, while enabling bacterial growth and systemic dissemination via embolism (Carrizosa *et al.*, 1978, Durack, 1975, Ferguson *et al.*, 1986a, Moreillon & Que, 2004).

Early studies failed to identify correlations between vegetation characteristics on echocardiograms and morbidity and mortality in infective endocarditis (De Castro *et al.*, 1997, Roder *et al.*, 1997, Steckelberg *et al.*, 1991). However, more recent studies have identified relationships between the presence of vegetations, vegetation size and vegetation mobility and the risk of emboli, valvular regurgitation and death (Cabell *et al.*, 2001, Di Salvo *et al.*, 2001, Fabri *et al.*, 2006, Martin-Davila *et al.*, 2005,

Wallace *et al.*, 2002). On meta-analysis, vegetation size greater than 10 mm was associated with embolism and need for surgery in infective endocarditis (Tischler & Vaitkus, 1997). Notably, *S. aureus* infective endocarditis is associated with large vegetations, possibly accounting for its association with high embolism and mortality rates (Fowler *et al.*, 2005b, Nadji *et al.*, 2005, Vilacosta *et al.*, 2002). Vegetations may therefore represent important therapeutic targets in infective endocarditis.

1.4.2 Vegetation composition

Light and electron microscopy of sterile vegetations from rabbit models of infective endocarditis demonstrate that they are predominantly composed of platelets in association with fibrin, leucocytes and erythrocytes (Carrizosa *et al.*, 1978, Durack, 1975, Ferguson *et al.*, 1986b). Bacteria are able to colonise sterile vegetations within 30 min of inoculation, with streptococci phagocytosed within monocytes and macrophages, and staphylococci mainly adherent to the irregular vegetation surface (Durack, 1975).

Approximately 3-6 h post-inoculation, bacteria replicate within vegetations in areas devoid of leucocytes (Durack, 1975, Ferguson *et al.*, 1986a, Ferguson *et al.*, 1986b). They aggregate in round colonies that are uniformly enclosed by protective fibrin layers and are dispersed amongst the reticular platelet-fibrin matrix (Carrizosa *et al.*, 1978, Durack, 1975, Ferguson *et al.*, 1986a). Activated platelets have been identified within infected vegetations, mainly at the interface of the vegetation surface and circulating blood (Rouzet *et al.*, 2008). Accumulation of successive layers of platelets, fibrin and bacteria leads to progressive enlargement of the vegetation. Healing eventually occurs by vegetation endothelialisation, phagocytosis, calcification and fibrous organisation, which are all enhanced by antimicrobial therapy (Durack, 1975).

1.4.3 Vegetation formation

The exact mechanism of vegetation formation in infective endocarditis is complex and still not fully understood. However, it is commonly thought that vegetations develop following bacterial, viral or fungal infection of sterile thrombi (Figure 1.3A) (Bashore *et al.*, 2006). Following microbial adherence to and incorporation within vegetations, monocytes are recruited by inter-cellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and secrete chemokines and proinflammatory cytokines, leading to tissue destruction (Chorianopoulos *et al.*, 2009, Heying *et al.*, 2007). Monocytes and endothelial cells also liberate tissue factor, resulting in platelet activation, increased fibrin generation and vegetation propagation (Moreillon *et al.*, 2002, Veltrop *et al.*, 2000).

Vegetation formation may also be initiated by bacterial attachment to plasma proteins coating native or prosthetic valvular tissue, followed by leucocyte and platelet recruitment (Figure 1.3B) (Bryers, 2008). Alternatively, preformed bacterium-platelet aggregates can adhere to cardiac valves (Figure 1.3C). It is not known at present whether platelets or bacteria are recruited to the valvular endothelium first (Chorianopoulos *et al.*, 2009). Either way, it is clear that platelet-bacterial-(sub)endothelial interactions contribute to vegetation formation.

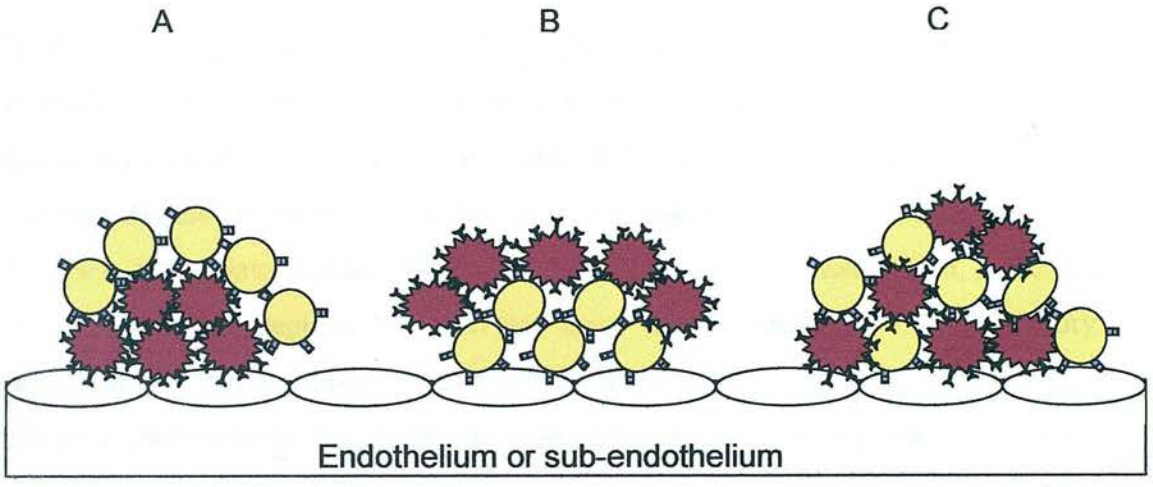

Figure 1.3. Possible mechanisms for bacterium and platelet attachment to cardiac valves. Bacteria are depicted in yellow, platelets in maroon. Platelets can adhere to (sub)-endothelium first and then recruit bacteria (A). Alternatively, bacteria can initially attach to cardiac valves and then recruit platelets (B). It is also feasible that bacterium-platelet aggregates form in the bloodstream and subsequently adhere to the valvular (sub)-endothelium (C).

1.4.3.1 Platelet-(sub)endothelium interactions

Platelets can translocate and roll on the endothelial surface, but platelet-endothelial interactions are usually prevented by the natural anticoagulant state of the vascular endothelium conferred by mediators such as nitric oxide, prostacyclin, heparan sulphate, thrombomodulin, glycocalyx, protein C and the ecto-ADPase CD39 (Chen & Lopez, 2005, Gawaz *et al.*, 2005). However, endothelial inflammation, as occurs in congenital and degenerative valvular heart disease, disables these inhibitory mechanisms (Chen & Lopez, 2005, Moreillon *et al.*, 2002). Platelets can then bind inflamed endothelium via GPIb or P-selectin glycoprotein ligand-1 (PSGL-1) binding to P-selectin, GPIb binding to vWF or GPIIb/IIIa adhering to vWF, fibrinogen or fibronectin (Figure 1.4A) (Chen & Lopez, 2005, Gawaz *et al.*, 2005).

Turbulent blood flow and inflammation from regurgitant or stenotic valves lead to endothelial denudation, exposing the subendothelial matrix proteins collagen and vWF (Moreillon *et al.*, 2002). Under the high shear conditions present *in vivo*, platelets form reversible bonds to these ligands via glycoprotein (GP)VI and GPIb receptors, respectively (Figure 1.4B) (Chen & Lopez, 2005, Gawaz *et al.*, 2005). Firm platelet binding to the subendothelium requires the formation of stronger low shear bonds between platelet receptors $\alpha_5\beta_1$, $\alpha_2\beta_1$, GPIIb/IIIa and their respective ligands (Figure 1.4B) (Chen & Lopez, 2005, Gawaz *et al.*, 2005).

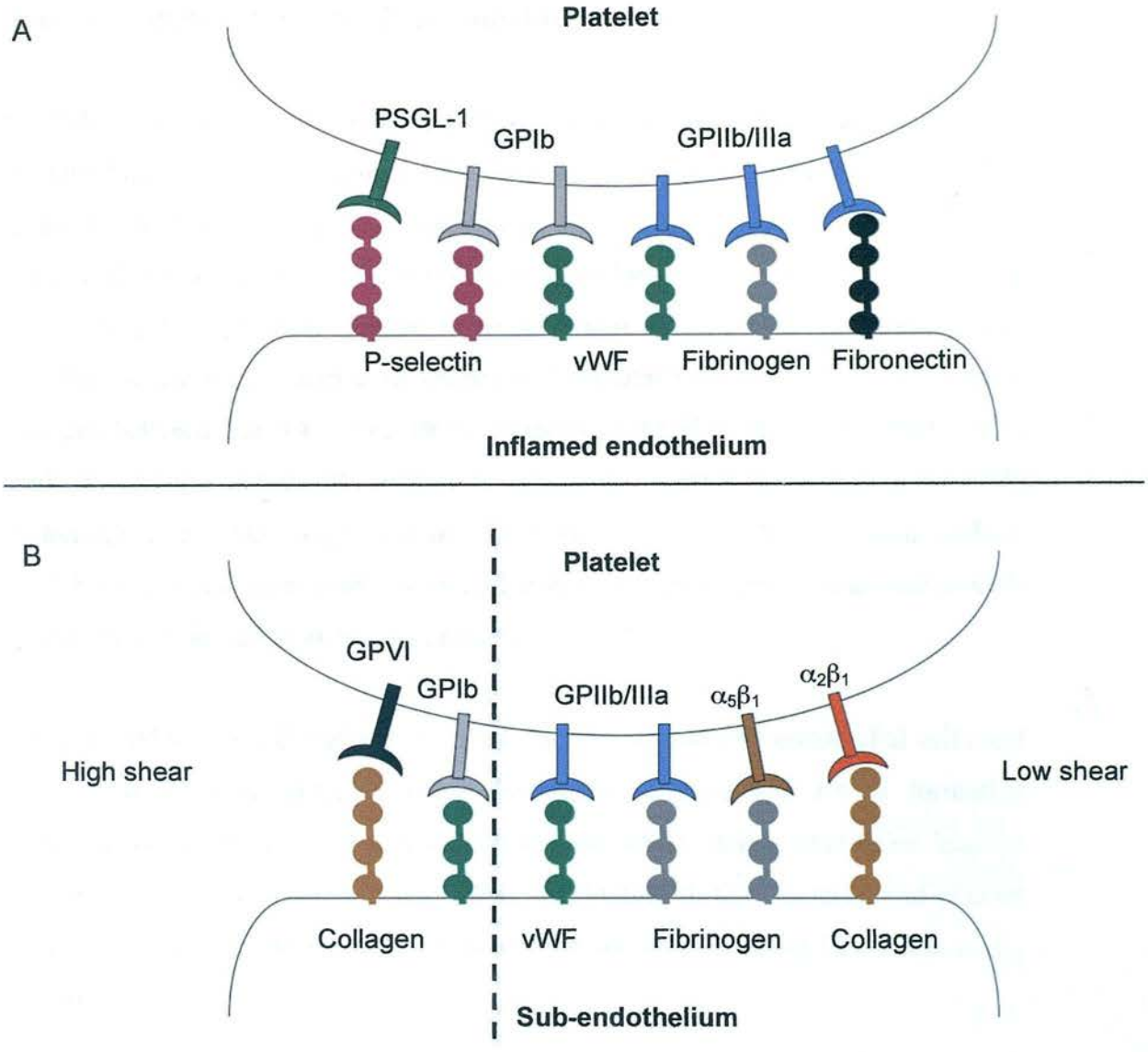

Figure 1.4. Platelet interactions with inflamed vascular endothelium and subendothelium resulting in the formation of thrombi on the valvular surface. Platelet receptors P-selectin glycoprotein ligand 1 (PSGL 1) (green), GPIb (grey) and GPIIb/IIIa (blue) are able to bind inflamed endothelium via their respective ligands (A). Platelet-subendothelium interactions under high shear involve GPVI (black) binding collagen (brown) and GPIb binding von Willebrand factor (vWF) (green) (B). At low shear, GPIIb/IIIa, $\alpha_5\beta_1$ (brown) and $\alpha_2\beta_1$ (red) bind the sub-endothelium via vWF, fibrinogen (grey) and collagen. Other extracellular matrix proteins include P-selectin (red) and fibronectin (black).

1.4.3.2 Bacterial-(sub)endothelium interactions

The ability of bacteria to adhere directly to valvular tissue, in particular undamaged endothelium, increases virulence and their ability to cause infective endocarditis (Chorianopoulos *et al.*, 2009, Vriesema *et al.*, 2000). *S. aureus* binds vascular subendothelium via a SpA-vWF interaction under low shear, or through Cna binding collagen under high shear (Figure 1.5A) (Chavakis *et al.*, 2005). *S. aureus* is also capable of adhering directly to undamaged endothelium, mainly via fibronectin bridges between the $A4^{+16}$ and BCD domains of FnBPA, and to a lesser extent FnBPB, and the endothelial integrin receptor $\alpha_5\beta_1$ under low shear (Figure 1.5B) (Chavakis *et al.*, 2005, Heying *et al.*, 2007, Piroth *et al.*, 2008). Cell wall teichoic acid has also been implicated in endothelial binding and virulence in animal models of infective endocarditis (Chorianopoulos *et al.*, 2009).

Once bound to the endothelium, *S. aureus* is phagocytosed by endothelial cells and releases α-toxin, resulting in valve destruction, abscess and fistula formation (Chorianopoulos *et al.*, 2009, Sinha & Herrmann, 2005). Valve destruction leads to the exposure of the subendothelial matrix, facilitating further bacterial and platelet recruitment, and resulting in propagation of the infected thrombus (Vriesema *et al.*, 2000).

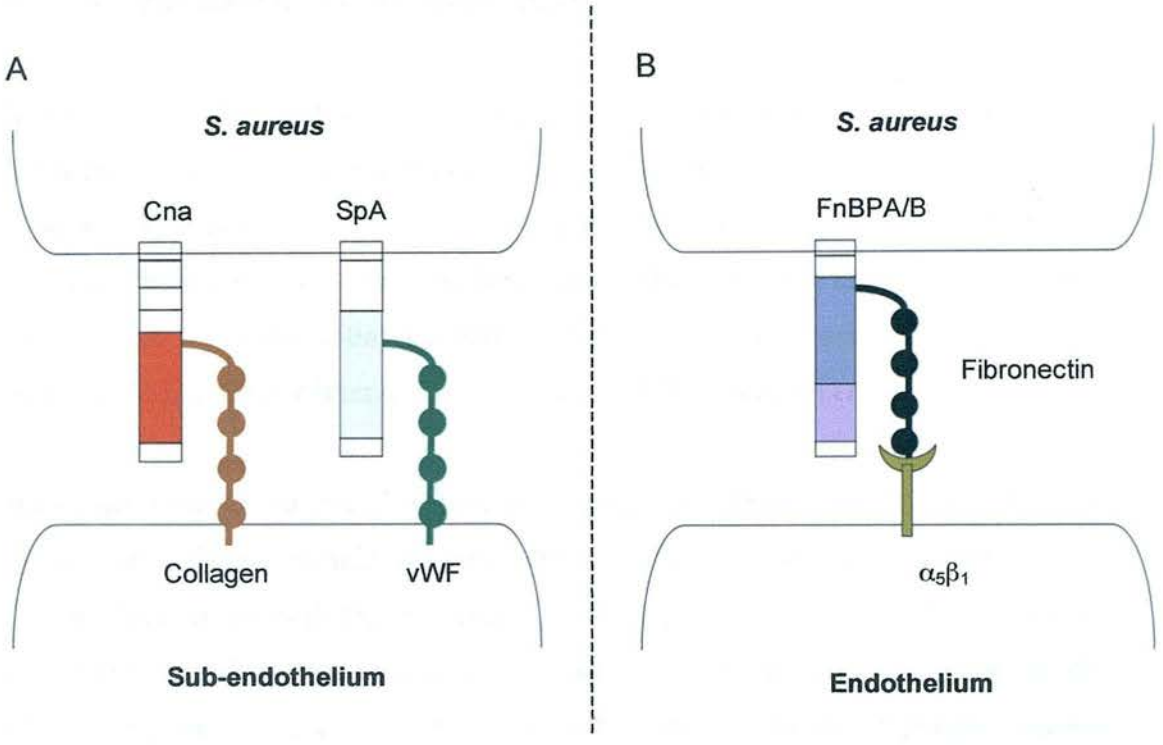

Figure 1.5. *Staphylococcus aureus* interactions with the sub-endothelium and endothelium contributing to valve colonisation in infective endocarditis. Collagen (brown) and von Willebrand factor (vWF) (green) exposed on denuded sub-endothelium can bind *S. aureus* via collagen adhesion (Cna) and Staphylococcal protein A (SpA), respectively (A). *S. aureus* is also able to bind integrins expressed on intact endothelium via fibronectin (black) bridges from the $A4^{+16}$ or BCD domains of fibronectin-binding proteins A or B (FnBPA/FnBPB) and the $\alpha_5\beta_1$ receptor (olive green) (B).

1.4.3.3 Bacterium-platelet interactions

It is widely considered that the interaction between bacteria and platelets is central to the pathogenesis of infective endocarditis (Pawar *et al.*, 2004, Sullam *et al.*, 1996). However, it was only recently that the first steps were taken in determining the molecular basis for this interaction. Bacterium-induced platelet aggregation is a two-step process, where the initial reversible bacterium-platelet adhesion is followed by a more stable irreversible interaction (Bayer *et al.*, 1995, Yeaman *et al.*, 1992c).

Bacterium-induced platelet activation and aggregation are mediated by the formation of IgG and plasma protein bridges between MSCRAMMs and platelet Fc and glycoprotein receptors (Fitzgerald *et al.*, 2006a). Such interactions result in 'inside-out' platelet activation via tyrosine kinase-dependent pathways, mediating the change in platelet shape from discoid to spherical, the release of platelet granule contents, and a conformational change and increase in the number of GPIIb/IIIa receptors, enabling fibrinogen binding and 'outside-in' platelet activation and recruitment (Figure 1.6) (Davi & Patrono, 2007, Fitzgerald *et al.*, 2006a). Thrombus formation is then mediated by cross-linking of GPIIb/IIIa on adjacent platelets via the dimeric fibrinogen molecule, leading to vegetation development (Figure 1.6) (Fitzgerald *et al.*, 2006a).

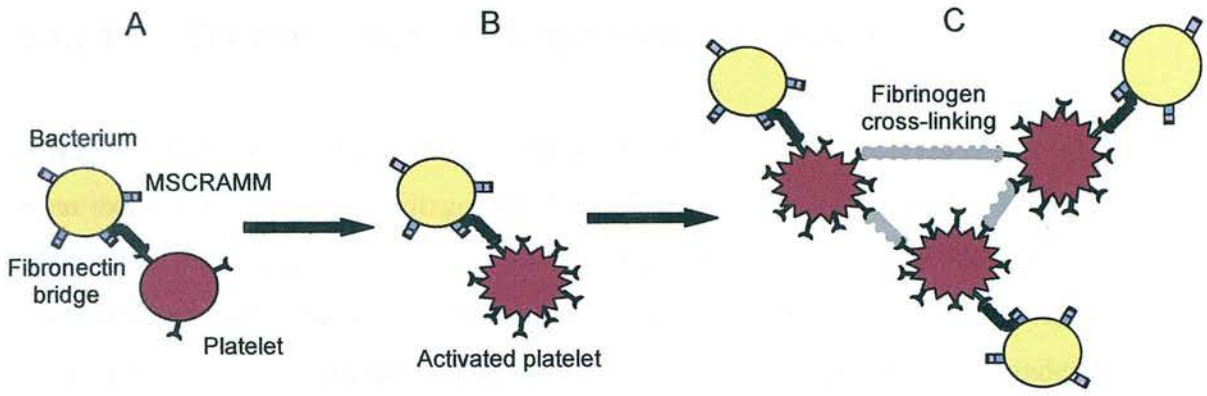

Figure 1.6. The mechanism of bacterium-induced platelet aggregation. Following bacterial adhesion to platelets via fibronectin or fibrinogen bridges between microbial surface components recognising adhesive matrix molecules (MSCRAMMs) and the platelet GPIIb/IIIa receptor (A), platelet activation occurs, resulting in a change of platelet shape from discoid to spherical and increased expression of GPIIb/IIIa (B). The activated GPIIb/IIIa receptors are able to bind soluble fibrinogen in plasma which cross-links, leading to platelet aggregation and thrombus formation (C). Adapted from (Fitzgerald *et al.*, 2006a).

1.4.3.3.1 The mechanisms of *Streptococcus*-platelet interactions

A number of organisms have the capacity to induce platelet aggregation and this has been extensively reviewed (Fitzgerald *et al.*, 2006a). The mechanisms of platelet aggregation induced by viridans streptococci were the first to be partially characterised. Early studies implicated *S. sanguinis* serine-rich protein A (SrpA) and *S. gordonii* Hsa and GspB interacting with the platelet GPIb receptor, independently of vWF (Kerrigan *et al.*, 2002, Plummer *et al.*, 2005, Takamatsu *et al.*, 2005). However, McNicol *et al* recently identified a role for vWF in *S. sanguinis*-induced platelet aggregation, as platelet aggregation did not occur when platelets were suspended in cryosupernatant depleted of vWF (McNicol *et al.*, 2007). Other postulated mechanisms of streptococcus-induced platelet aggregation include binding via complement, by a fibrinogen bridge to the GPIIb/IIIa platelet receptor, via platelet-aggregation-associated protein (PAAP) binding to collagen and by an IgG-FcγRIIa interaction (Ford *et al.*, 1997, Ford *et al.*, 1996, Herzberg *et al.*, 1992, Pampolina & McNicol, 2005, Sullam *et al.*, 1988).

1.4.3.3.2 The mechanisms of *S. aureus*-platelet interactions

Early research into streptococcus-induced platelet aggregation served as a model for elucidation of the mechanisms of *S. aureus*-platelet interactions. The initial binding of *S. aureus* to platelets can either occur directly via MSCRAMMs, or indirectly via bridging plasma proteins such as fibrinogen or fibronectin binding to the platelet GPIIb/IIIa receptor (Figures 1.7A and 1.7B) (Fitzgerald *et al.*, 2006b, O'Brien *et al.*, 2002a). An alternative slower fibrinogen-independent mechanism utilising complement has also been identified (Figure 1.7C) (Loughman *et al.*, 2005, Miajlovic *et al.*, 2007, O'Brien *et al.*, 2002a). Furthermore, SpA is capable of inducing platelet activation via methods similar to those observed for streptococci, including the use of complement bound to the gC1qR/p33 complement receptor, vWF to the GPIb receptor or the Fc portion of IgG to the FcγRIIa platelet receptor (Figure 1.7D) (Hartleib *et al.*, 2000, Nguyen *et al.*, 2000, O'Brien *et al.*, 2002a).

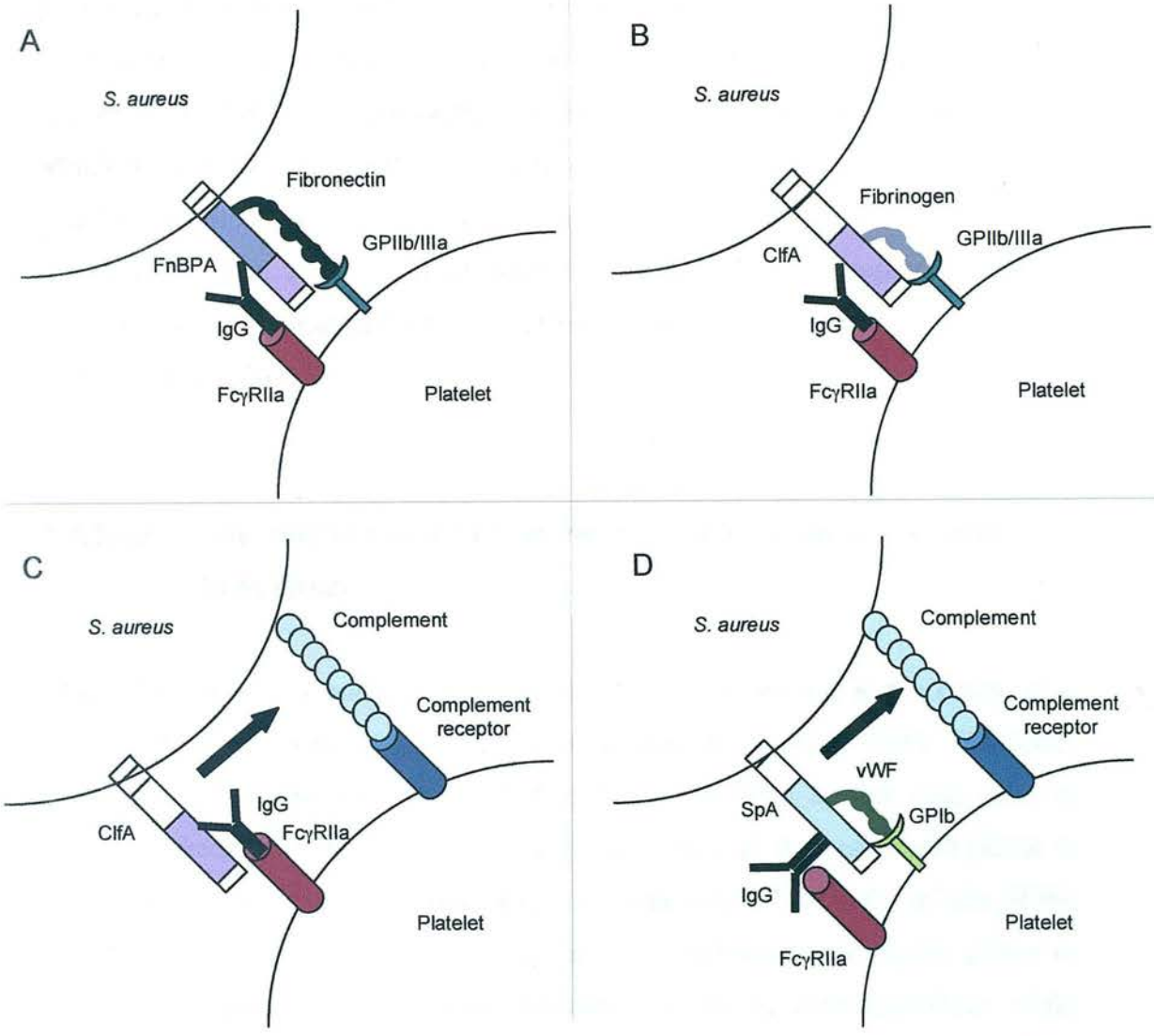

Figure 1.7. The mechanisms of *S. aureus*-induced platelet aggregation. Initial rapid bacterium-platelet adhesion is mediated by linkage of microbial surface components recognising adhesive matrix molecules (MSCRAMMs) to GPIIb/IIIa via either fibronectin (A) or fibrinogen (B) bridges. Platelet activation and aggregation occur when IgG specific for MSCRAMMs binds irreversibly to the platelet FcγRIIa receptor (A and B). Alternatively, slower activation can be facilitated through complement (C). Staphylococcal protein A (SpA) is capable of mediating platelet aggregation by various methods, including the use of complement, by binding to the Fc portion of IgG or via a von Willebrand factor (vWF) bridge to the GPIb receptor (D). FnBPA, fibronectin-binding protein A; ClfA, clumping factor A. Adapted from (Fitzgerald *et al.*, 2006a, Fitzgerald *et al.*, 2006b, O'Brien *et al.*, 2002a).

MSCRAMM binding to GPIIb/IIIa or GPIb is rapid, saturable and reversible, but insufficient to independently cause platelet activation and thrombus formation (Bayer *et al.*, 1995). Cross-linking of the Fc portion of antibody specific for the MSCRAMM mediating platelet adherence to the platelet FcγRIIa receptor, facilitates platelet activation and aggregation (Fitzgerald *et al.*, 2006a, Sjobring *et al.*, 2002) (Figures 1.7A and 1.7B). Indeed, inhibition of the FcγRIIa receptor completely abrogates *S. aureus*-induced platelet activation *in vitro* (Fitzgerald *et al.*, 2006b, Sjobring *et al.*, 2002).

1.4.3.3.3 The mechanisms of *S. aureus*-platelet interactions under high shear

Blood flow *in vivo* is pulsatile and not uniform as it is highest at the centre of a vessel with minimum flow adjacent to the vessel wall (Kroll *et al.*, 1996). This leads to shearing effects between layers of fluid that are moving against each other at different speeds, with the greatest shearing effects seen at the vessel wall (Kroll *et al.*, 1996). Under high shear, erythrocytes and leucocytes flow in the middle of the vessel, while platelets are at the periphery and in a better position to rapidly adhere to cardiac valves (Kerrigan *et al.*, 2007). Coronary vessels are under high shear, while the valve surface is more complex due to the additional presence of turbulent blood flow (Kerrigan *et al.*, 2007).

Studies analysing *S. aureus*-platelet interactions under varying shear have reported that fewer and smaller *S. aureus*-platelet thrombi are formed under high shear as compared to low shear conditions (Johnson & Ross, 2008). Furthermore, *S. aureus*-platelet binding varies with both shear and bacterial growth phase *in vitro* (George *et al.*, 2007, Pawar *et al.*, 2004). Binding is greatest at low shear for exponential phase strains and at high shear for stationary phase cultures, reflecting the maximal expression of FnBPs at the exponential phase of growth and increased expression of ClfA during the stationary phase of growth (George *et al.*, 2007). High shear *S. aureus*-platelet interactions at both stationary and exponential phases of growth

involve a two-step process, where the initial adhesion is mediated by SpA binding to GPIb via vWF (Fig. 1.7D), increasing contact time to enable the slower yet stronger bond between ClfA and the platelet surface to form (George *et al.*, 2007, George *et al.*, 2006, Kerrigan *et al.*, 2007, Pawar *et al.*, 2004). Recently however, high shear *S. aureus*-platelet interactions mediated by FnBPs binding fibrinogen or fibronectin during the early exponential phase of growth have been described (George *et al.*, 2007).

1.5 The role of platelets in sepsis and infective endocarditis

1.5.1 Platelet function in sepsis

Even in the absence of infective endocarditis, sepsis is a pro-thrombotic state with upregulation of pro-coagulant and reduced anti-coagulant activity (Schouten *et al.*, 2008). Platelet activation is increased in sepsis, as measured by GPIIb/IIIa receptor activity, thrombospondin, platelet P-selectin expression and platelet monocyte aggregate (PMA) formation (Gawaz *et al.*, 1995, Ogura *et al.*, 2001, Russwurm *et al.*, 2002).

Bacteria induce platelet activation by stimulating the production of pro-inflammatory cytokines such as interleukin-1, tumour necrosis factor-α, C-reactive protein (CRP) and the release of endotoxin by Gram-negative organisms (Levi *et al.*, 2003). In addition, they activate the coagulation cascade by stimulating the release of tissue factor, impairing the action of physiological anticoagulants (e.g. anti-thrombin, protein C) and by inducing fibrinolysis via plasminogen activation (Levi *et al.*, 2003, Schouten *et al.*, 2008). Although activation of the host coagulation system forms part of the innate immune defence, an exaggerated response can lead to disease pathogenesis (Schouten *et al.*, 2008, Sjobring *et al.*, 2002). Specifically, an uncontrolled coagulation cascade can lead to a generalised consumption coagulopathy, which contributes to the development of thrombocytopaenia,

hypovolaemic shock, disseminated intravascular coagulation (DIC) and multi-organ failure in sepsis (Gawaz *et al.*, 1997, Schouten *et al.*, 2008).

The initial recruitment of platelets to the site of infection appears to be protective and forms part of the immune response (Sjobring *et al.*, 2002). Platelets exhibit pro-inflammatory effects via the expression of IgG-Fc and complement receptors for opsonisation, release of interleukin-1β, generation of toxic oxygen free radicals and enhancement of leucocyte recruitment to the site of sepsis (Yeaman, 1997). Platelets increase neutrophil respiratory burst in response to opsonised and non-opsonised organisms including *S. aureus* (Miedzobrodzki *et al.*, 2008), and can engulf microorganisms, although there is no evidence of bacterial killing (White, 2006, Youssefian *et al.*, 2002). In addition, platelets exert antimicrobial effects via the release of PMPs (Yeaman, 1997).

1.5.2 Platelet microbicidal proteins (PMPs)

PMPs are small cationic proteins released from platelet α-granules at sites of endovascular damage or microbial colonisation (Fowler *et al.*, 2004). Their release is stimulated by thrombin or bacteria activating platelet receptors $P2X_1$ and $P2Y_{12}$ (Trier *et al.*, 2008). PMPs disrupt bacterial membranes and intracellular macromolecular synthesis, while enhancing the lymphocyte response to infection (Kupferwasser *et al.*, 2002, Tang *et al.*, 2002). They exert microbicidal activity against a wide range of organisms including staphylococci, viridans streptococci, *Escherichia coli*, and *Candida spp* (Tang *et al.*, 2002, Yeaman *et al.*, 1994). To date, at least 7 PMPs have been identified from resting and activated human platelets, the most prevalent being connective tissue-activating peptide 3 and platelet factor-4 (Tang *et al.*, 2002).

PMPs reduce *S. aureus* adherence to platelets and sterile thrombi and reduce bacterial proliferation within established vegetations (Mercier *et al.*, 2004, Yeaman *et al.*, 1994). Furthermore, PMPs and antibiotics act synergistically; PMPs potentiate

et al., 1994). Furthermore, PMPs and antibiotics act synergistically; PMPs potentiate the microbicidal activity of vancomycin and penicillin, while antibiotics increase the ability of PMPs to reduce bacterium-platelet binding *in vitro* (Mercier *et al.*, 2004, Yeaman *et al.*, 1994, Yeaman *et al.*, 1992a).

Staphylococci and viridans streptococci can acquire PMP-resistance which confers a survival advantage, increases microbial virulence and increases the propensity to cause infective endocarditis, particularly in patients with intravascular device infection (Fowler *et al.*, 2000, Kupferwasser *et al.*, 2002, Wu *et al.*, 1994). In a study analysing 60 *S. aureus* clinical isolates, 83% of strains isolated from cases of infective endocarditis were PMP-resistant, in comparison to one-third of strains implicated in soft tissue sepsis (Bayer *et al.*, 1998). Notably, Yeaman *et al* could not identify a correlation between the ability of *S. aureus* strains to induce platelet aggregation and exhibit PMP resistance *in vitro*, suggesting these phenomena are independent of one another (Yeaman *et al.*, 1992b).

1.5.3 The role of platelets in the development of infective endocarditis

In theory, platelets could have a conflicting role in the pathogenesis of infective endocarditis. They form part of the immune response, but can also interact with bacteria to form thrombi and vegetations (see Section 1.4.3). Trier *et al* provided evidence that the outcome of bacterium-platelet interactions may depend on the ratio of platelets to bacteria, as platelet:*S. aureus* ratios of 10:1 or greater were associated with reduced bacterial viability (Trier *et al.*, 2008).

Thrombocytopenia, often in association with DIC, is present in 14% to 18% of patients with infective endocarditis, and has been attributed to the development of antibodies against the platelet GPIIb/IIIa receptor (Bouza *et al.*, 2001, Mouly *et al.*, 2002). However, Hogevik *et al* could not identify a correlation between platelet count and a diagnosis of infective endocarditis (Hogevik *et al.*, 1997). Thrombocytopenia has been associated with increased intra-vegetation bacterial

proliferation in an experimental rabbit model (Sullam *et al.*, 1993), and a non-significant trend towards increased mortality in a study of 89 patients with infective endocarditis (Mouly *et al.*, 2002). Both of these studies suggest that platelets play a predominant role in immune defence rather than the development of infective endocarditis, although it is not clear whether these are causal relationships.

A number of factors may affect platelet function in infective endocarditis and should be taken into consideration in platelet activation studies. For example, anticoagulants increase platelet activation (Harding *et al.*, 2006a, Mieszczak & Winther, 1996) and multi-organ failure is associated with reduced platelet activation (Gawaz *et al.*, 1995, Russwurm *et al.*, 2002). Furthermore, increased platelet activation has been observed in patients with mitral stenosis and mitral regurgitation (Chen *et al.*, 2004, Tse *et al.*, 1997), while either increased (Chirkov *et al.*, 2002, Goldsmith *et al.*, 2000) or reduced (O'Brien *et al.*, 1995, Pareti *et al.*, 2000) platelet activation and aggregation have been observed in aortic stenosis; the latter is thought to be due to enhanced cleavage of vWF multimers, similar to that observed in type 2b von Willebrand's disease. Most studies have observed increased platelet activation in patients following cardiac valve replacement, particularly in those receiving bileaflet mechanical valves (Koppensteiner *et al.*, 1991, Leguyader *et al.*, 2006, Maugeri *et al.*, 2000).

1.6 The effect of platelet receptor genetic polymorphisms on platelet function and susceptibility to disease

Platelet GPIIb/IIIa, GPIb and FcγRIIa receptors are involved in *S. aureus*-induced platelet activation and aggregation (see Section 1.4.3.3.2 and Figure 1.7) (Fitzgerald *et al.*, 2006a). Polymorphisms of these receptors increase the sensitivity to platelet aggregation induced by pharmacological agonists and have been associated with vascular and immune disease (Meisel *et al.*, 2004, van der Pol & van de Winkel, 1998), and could therefore influence susceptibility to infective endocarditis.

1.6.1 Polymorphisms of the platelet GPIIb/IIIa receptor

GPIIb/IIIa or CD41/CD61 is a heterodimeric integrin protein linked noncovalently by disulphide bridges (Phillips *et al.*, 1988). It is capable of binding fibrinogen, fibronectin, vWF and vitronectin (Meisel *et al.*, 2004). GPIIb/IIIa is the most abundant platelet receptor, with 80,000 copies per platelet, and genes for both components have been mapped to chromosome 17q21.32 (Meisel *et al.*, 2004).

The GPIIIa $PI^{A1/A2}$ polymorphism was first described in 1989, and consists of a T to C substitution at nucleotide 1565 in exon 2 of the GPIIIa gene, resulting in a leucine to proline substitution at residue 33 (Newman *et al.*, 1989). Approximately 72% of Caucasians are homozygous for PI^{A1}, 26% are heterozygous and 2% are homozygous for PI^{A2} (Table 1.4) (Laule *et al.*, 1999, Newman *et al.*, 1989). The PI^{A2} allele is thought to be dominant (O'Halloran *et al.*, 2006).

1.6.1.1 Correlations between the GPIIIa $PI^{A1/A2}$ polymorphism and platelet aggregation

PI^{A2} alleles are generally been thought to confer a pro-aggregatory phenotype independent of GPIIb/IIIa receptor expression levels (Michelson *et al.*, 2000, O'Halloran *et al.*, 2006). Ligand-engagement of platelet GPIIb/IIIa receptors is associated with increased phosphatase PP2A activity, leading to increased activation of the extracellular-signal regulated kinase-2 signalling pathway and enhanced myosin light chain dephosphorylation (Vijayan & Bray, 2006, Wang *et al.*, 2008). These actions are more pronounced in the PI^{A2} variant, collectively resulting in enhanced platelet activation and aggregation (Vijayan & Bray, 2006).

Table 1.4. The observed frequencies of polymorphisms of platelet receptors GPIIb/IIIa, GPIb and FcγRIIa in healthy Caucasians. Data adapted from (Baker *et al.*, 2001, Jilma-Stohlawetz *et al.*, 2003, Laule *et al.*, 1999, Lehrnbecher *et al.*, 1999, Newman *et al.*, 1989)

Platelet Receptor	Polymorphisms	Genotypes	Frequencies
GPIIb/IIIa	$PI^{A1/A2}$	$PI^{A1/A1}$	72%
		$PI^{A1/A2}$	26%
		$PI^{A2/A2}$	2%
GPIb	VNTR	D/D	0.5-4.4%
		D/C	8.8-10.5%
		C/C	66.9-83.9%
		D/B	1.9%
		C/B	2.1-18.9%
		B/B	1.0-1.3%
	HPA-2	2a/2a	84.9%
		2a/2b	13.7%
		2b/2b	1.4%
	Kozak sequence	T/T	75.5%
		T/C	22.3%
		C/C	2.2%
FcγRIIa	H131R	R/R	25%
		H/R	44%
		H/H	31%

There have been conflicting results from studies examining the effects of the $PI^{A1/A2}$ polymorphism on susceptibility to platelet aggregation induced by pharmacological agonists. One of the first studies to analyse the influence of the polymorphism was the 1,422 subject Framingham Offspring Study (Feng *et al.*, 1999). The investigators identified an increase in susceptibility to epinephrine- and no alteration in response to adenosine diphosphate (ADP)-induced aggregation in the presence of $PI^{A1/A2}$ compared to $PI^{A1/A1}$ platelets. There was no change for PI^{A2} homozygotes, but this was attributed to the low prevalence of this genotype. Michelson *et al* identified a larger proportion of individuals with a $PI^{A2/A2}$ genotype, which was associated with an increase in baseline platelet P-selectin expression (Michelson *et al.*, 2000). ADP stimulation in this group led to significantly raised P-selectin expression, GPIIb/IIIa activation and fibrinogen binding, correlating with increased platelet reactivity (Michelson *et al.*, 2000). In contrast, Frey *et al* did not identify any $PI^{A1/A2}$-dependent difference in platelet function in response to either ADP or epinephrine (Frey *et al.*, 2003). Conversely, other investigators have demonstrated increased platelet aggregation in PI^{A1} homozygotes in response to the protease-activated receptor-1 (PAR-1) agonist SFLLRN, ADP arachidonic acid, and the thromboxane A2 analogue U46619 (Andrioli *et al.*, 2000, Lasne *et al.*, 1997).

1.6.1.2 Associations of the GPIIIa $PI^{A1/A2}$ polymorphism with clinical disease

The PI^{A2} allele correlates with the development of acute coronary syndromes (ACS), premature myocardial infarction (MI) and ischaemic cerebrovascular disease, particularly in otherwise low-risk patients (Carter *et al.*, 1998, Gardemann *et al.*, 1998, Saidi *et al.*, 2008). However, the influence of the PI^{A2} allele on in-stent restenosis is unclear (Kastrati *et al.*, 2000, Le Hello *et al.*, 2007). Presence of a PI^{A2} allele is associated with arterial thrombosis in patients with immune-mediated heparin-induced thrombocytopaenia (Harris *et al.*, 2008). No correlation was identified between $PI^{A1/A2}$ genotype and the development of intravascular device infection (Musher *et al.*, 2002).

The most recent review of the GPIIb/IIIa polymorphism analysed the role of this genetic variant in patients with coronary artery disease and ACS (34 studies), coronary stent restenosis post-percutaneous coronary intervention (PCI) (6 studies) and cerebrovascular disease (6 studies) (Meisel *et al.*, 2004). Only a weak association was identified between the $Pl^{A1/A2}$ polymorphism and susceptibility to these conditions (Meisel *et al.*, 2004).

1.6.2 Polymorphisms of the platelet GPIb receptor

The GPIb-IX-V complex consists of four subunits, GPIbα, GPIbβ, GPIX and GPV, encoded by genes on chromosomes 17p12, 22q11.2 and 3, respectively (Meisel *et al.*, 2004, Ware, 1998). GPIbα is the active component, binding to vWF at its N-terminus (Ware, 1998). Three polymorphisms have been described for the active ligand-binding site of GPIb with frequencies outlined in Table 1.4 (Meisel *et al.*, 2004, Ware, 1998).

The variable number of tandem repeat (VNTR) allele codes for 0, 1, 2, 3 or 4 repeats of a 13 amino acid sequence, designated E, D, C, B and A respectively, that facilitates extension of the active ligand-binding site of GPIbα from the platelet surface (Lopez *et al.*, 1992, Ozelo *et al.*, 2004a). The presence of a C to T substitution at nucleotide 434 of the GPIbα gene, results in an amino acid change from threonine to methionine at residue 145, conferring a change from human platelet antigen (HPA)-2a to 2b (Meisel *et al.*, 2004). The HPA-2 variant is in linkage disequilibrium with VNTR, with HPA-2a associated with VNTR C/D and HPA-2b with VNTR A/B (Meisel *et al.*, 2004). HPA-2b is thought to exert a dominant effect, with 84.9% homozygous for HPA-2a, 1.4% homozygous for HPA-2b and 13.7% of individuals heterozygous (Table 1.4) (Baker *et al.*, 2001, Jilma-Stohlawetz *et al.*, 2003).

The third polymorphism, known as a Kozak sequence polymorphism, involves a T to C substitution 5 positions downstream from the GPIbα initiator and the C allele is

proposed to be dominant (Afshar-Kharghan *et al.*, 1999). Just over three-quarters of the population are homozygous for T/T, 22.3% heterozygous and 2.2% homozygous for C/C (Table 1.4) (Baker *et al.*, 2001, Jilma-Stohlawetz *et al.*, 2003).

1.6.2.1 Correlations between GPIb receptor polymorphisms and platelet aggregation

Most *in vitro* studies have demonstrated that neither the number of surface GPIbα molecules, nor the efficiency of vWF binding are affected by the presence of HPA-2 polymorphisms (Jilma-Stohlawetz *et al.*, 2003, Li *et al.*, 2000a), whilst Ulrichts *et al* identified increased vWF binding in HPA-2a platelets (Ulrichts *et al.*, 2003). HPA-2b/VNTR-A platelets have a slow rolling velocity at high shear, with no effect on vWF binding (Matsubara *et al.*, 2005). Conversely, platelet plug formation is significantly increased with VNTR C/D platelets (Jilma-Stohlawetz *et al.*, 2003). There have been conflicting reports as to the effect of the Kozak sequence variant on GPIb receptor expression and platelet plug formation (Afshar-Kharghan *et al.*, 1999, Jilma-Stohlawetz *et al.*, 2003).

1.6.2.2 Associations of GPIb receptor polymorphisms with clinical disease

The correlation between VNTR alleles and coronary artery disease is unclear as B, C and D alleles have all been implicated in MI (Gonzalez-Conejero *et al.*, 1998, Mikkelsson *et al.*, 2001, Ozelo *et al.*, 2004b). Further research has demonstrated that HPA-2b predisposes to cerebrovascular disease, but others do not corroborate these findings (Baker *et al.*, 2001, Carter *et al.*, 1998, Meisel *et al.*, 2004). The VNTR C/D and Kozak sequence T/C genotypes are both associated with intravascular catheter infection (Musher *et al.*, 2002).

A review investigated the impact of GPIb polymorphisms on clinical disease, including 14 studies investigating coronary artery disease, ACS and PCI, and 6 investigating cerebrovascular disease (Meisel *et al.*, 2004). Overall, there was no conclusive link between the presence of VNTR or HPA-2 alleles and coronary artery disease, although a recent meta-analysis identified an association of the HPA-2b allele with the development of cerebrovascular disease (Maguire *et al.*, 2008, Meisel *et al.*, 2004). The C allele of the Kozak sequence polymorphism is associated with increased risk of ACS, adverse events post-PCI, MI, cerebrovascular disease and experimental arterial thrombus formation, suggesting a possible role for this allele in acute thrombotic events (Baker *et al.*, 2001, Cadroy *et al.*, 2001, Meisel *et al.*, 2004).

1.6.3 Polymorphisms of the FcγRIIa receptor

FcγRIIa is a low affinity IgG-Fc receptor that exhibits stronger binding to multimeric as opposed to monomeric IgG (Lehrnbecher *et al.*, 1999). It is present on leucocytes where it contributes to phagocytosis of encapsulated bacteria and *S. aureus* opsonised with IgG_2 (Bredius *et al.*, 1993, van der Pol & van de Winkel, 1998), and is the only Fc receptor on platelets, numbering between 1,300 and 8,500 on the platelet surface (Chen *et al.*, 2003). A functional polymorphism resulting from a G to A point mutation, results in an arginine (R) to histidine (H) change at position 131 (van der Pol & van de Winkel, 1998). Approximately 25% of individuals are R/R, 31% H/H and 44% heterozygous, with codominant expression of both alleles (Table 1.4) (Lehrnbecher *et al.*, 1999).

1.6.3.1 Correlations between the FcγRIIa H131R polymorphism, IgG binding and platelet aggregation

The R131 allotype is associated with increased binding to mouse IgG_1, while the H131 allotype exhibits increased binding to human IgG_2 and IgG_3 (van der Pol & van de Winkel, 1998). The presence of at least one R allele significantly increases

ADP-induced antibody recognition of the GPIIb/IIIa-ligand binding site on human platelets (Chen *et al.*, 2003). However, H131R genotype has no effect on fibrinogen binding, pharmacological agonist-induced platelet aggregation or shear-induced platelet activation (Chen *et al.*, 2003).

1.6.3.2 Associations of the FcγRIIa H131R polymorphism with clinical disease

The H131 variant was associated with the development of systemic lupus erythematosus on meta-analysis (Lehrnbecher *et al.*, 1999). Furthermore, the heterozygous state was associated with the development of thromboembolic complications in patients with anti-phospholipid syndrome (Schallmoser *et al.*, 2005). Although individual studies have identified correlations between the presence of an R131 allele and susceptibility to invasive pneumococcal or meningococcal disease, there was no clear association on meta-analysis (Brouwer *et al.*, 2009). There was no relationship between H131R genotype and susceptibility to coronary artery disease in a recent large study (Karakas *et al.*, 2009). However, given the crucial role of the FcγRIIa receptor in *S. aureus*-mediated platelet aggregation, this polymorphism may influence susceptibility to infective endocarditis.

1.7 Treatments targeting platelet function in infective endocarditis

1.7.1 Effect of antibiotics on platelet function

Current treatment options for infection, namely antibiotics, also influence platelet function. For example, vancomycin and teicoplanin induce thrombocytopenia, possibly via autoimmune mechanisms, as affected individuals have increased serum anti-GPIIb/IIIa antibody titres (Garner *et al.*, 2005, Von Drygalski *et al.*, 2007). Furthermore, antibiotics at high concentrations can interact directly with platelet

phospholipid membranes and receptors, resulting in reduced bacterium-platelet and bacterium-vegetation adhesion in rabbit models of infective endocarditis, and reduced platelet activation and aggregation *in vitro* (Lowy *et al.*, 1983, Pastakia *et al.*, 1993, Shattil *et al.*, 1980).

Several studies have analysed the relationship between antibiotic therapy and embolic events in infective endocarditis. Most have found embolic rates to be reduced by antibiotic treatment (Di Salvo *et al.*, 2001, Steckelberg *et al.*, 1991, Thuny *et al.*, 2005, Vilacosta *et al.*, 2002), while others have found either increased (Roder *et al.*, 1997) or equivalent rates (Dickerman *et al.*, 2007, Fabri *et al.*, 2006) of embolism pre- and post-antimicrobial therapy. The incidence of septic emboli appears to be highest upon the initiation of treatment, but this probably reflects the fact that emboli are presenting features in 14% to 47% of cases of infective endocarditis (Dickerman *et al.*, 2007, Fabri *et al.*, 2006, Roder *et al.*, 1997). Overall, once antimicrobial therapy is commenced, the frequency of embolic events decreases, even when confounding factors such as surgery are taken into consideration (Dickerman *et al.*, 2007, Fabri *et al.*, 2006, Roder *et al.*, 1997, Steckelberg *et al.*, 1991, Thuny *et al.*, 2005).

Different classes of antibiotics have varying effects on platelet function. For example, increased vegetation size and embolic risk are associated with cephalosporin therapy, while treatment with penicillin or vancomycin are associated with reduction in vegetation size (Rohmann *et al.*, 1997). However, it is unclear whether these effects are by virtue of their antimicrobial or anti-platelet effects.

1.7.2 The role of anti-platelets in the treatment of infective endocarditis

Although the role of antibiotics in the treatment of infective endocarditis is well established, the role of anti-platelets is not clear (Habib *et al.*, 2009).

1.7.2.1 Results from experimental animal models of infective endocarditis

High concentrations of aspirin or salicylate reduce bacterium-platelet and bacterium-thrombus binding, bacterium-induced platelet aggregation, vegetation weight, vegetation and renal bacterial densities and renal infarction in animal models of *S. aureus* infective endocarditis (Kupferwasser *et al.*, 1999, Nicolau *et al.*, 1993). Reductions in vegetation weight alone have been observed with ticlopidine therapy, the combination of early aspirin or ticlopidine with vancomycin, or with dual anti-platelet therapy (Nicolau *et al.*, 1999, Nicolau *et al.*, 1998). However, aspirin was reported to have no effect on vegetation and infective endocarditis development induced by viridans streptococci in a rabbit model, suggesting that the beneficial effects of aspirin in infective endocarditis may be bacterial species specific (Levison *et al.*, 1977).

1.7.2.2 Results from human studies of infective endocarditis

A retrospective study investigating patients with tunnelled haemodialysis catheters, found a significant reduction in *S. aureus* bacteraemia rates in those on aspirin therapy (Sedlacek *et al.*, 2007). A small clinical trial of 9 patients with infective endocarditis identified a reduction in vegetation size, embolic events and mortality amongst those treated with aspirin (Taha *et al.*, 1992). Furthermore, Adler *et al* reported a case of *Serratia marcescens* neonatal infective endocarditis complicated by progressively enlarging vegetations that failed to respond to potent intravenous antibiotics, but improved with oral aspirin therapy (Adler *et al.*, 2004).

Unfortunately, the beneficial effects of aspirin observed in case series and animal models have not been consistently observed in larger studies. The double-blinded placebo-controlled Multi-centre Aspirin Trial in Infective Endocarditis (MATIE) study analysed the effect of treatment with 325 mg of aspirin daily in 115 patients with infective endocarditis (Chan *et al.*, 2003). There was no reduction in embolic

risk or mortality with early or late aspirin treatment, but there was an apparent trend towards increased haemorrhagic complications in those on long-term therapy (Chan *et al.*, 2008, Chan *et al.*, 2003). Conversely, in an observational study of 125 patients, Anavekar *et al* discovered that at least 6 months prior anti-platelet therapy with aspirin, clopidogrel, ticlopidine or dipyridamole, regardless of dose, resulted in significantly fewer emboli (Anavekar *et al.*, 2007). However, the results of this study should be interpreted with caution, as the investigators were limited by small study populations in each treatment group (Anavekar *et al.*, 2007).

Accordingly, recently published studies have investigated the effect of long-term anti-platelet therapy on outcome in infective endocarditis (Eisen *et al.*, 2009, Pepin *et al.*, 2009). A single-centre 16-year follow-up study discovered that prior anti-platelet therapy was associated with reduced mortality, particularly in non-*S. aureus* infective endocarditis, with no effect on embolism (Pepin *et al.*, 2009). In contrast, a retrospective study on 670 patients with *S. aureus* infective endocarditis determined that aspirin therapy was associated with decreased risk of embolism on univariate analysis alone, and correlated with reduced need for surgery during the index event (Eisen *et al.*, 2009).

1.7.2.3 Antimicrobial effects of anti-platelets

Some anti-platelet derivatives exhibit antimicrobial effects *in vitro*. Notably, these phenomena have mainly been observed with salicylate, a derivative of aspirin (acetylsalicylic acid) that lacks intrinsic anti-platelet activity (Kupferwasser *et al.*, 2003). Growth in the presence of salicylate abrogates bacterial growth, alters bacterial metabolism, modulates antimicrobial resistance and reduces attachment to valvular endothelium (Kupferwasser *et al.*, 1999, Price *et al.*, 2000, Riordan *et al.*, 2007). Furthermore, salicylate influences virulence factor expression by downregulating *S. aureus fnbA*, *fnbB*, *hla* (encoding α-toxin), *sarA* promoter and *agr* RNAIII expression, while upregulating exoenzyme and *spa* expression through σ^B-dependent mechanisms (Kupferwasser *et al.*, 2003, Price *et al.*, 2000, Riordan *et*

al., 2007). It is not known at present whether similar effects are observed with aspirin.

1.8 Conclusions

S. aureus infective endocarditis is a serious condition whose incidence has increased globally. However, many patients who contract this disease have no known risk factors, and it is not understood why some patients recover rapidly from bacteraemia, while others develop infective endocarditis. Considering the high morbidity and mortality rates associated with infective endocarditis, it is important to understand why it occurs as a complication of bacteraemia in some cases, but not others.

Recent evidence suggests that the outcome of infection may be related to both host and pathogen factors, but our knowledge of this is far from complete (Fowler *et al.*, 2005a, Lowy, 1998). For example, it is unclear whether bacterial factors which are implicated in platelet interactions *in vitro* and in animals, are also relevant to the pathogenesis of infective endocarditis in humans. In addition, the role of platelets in infective endocarditis is not known, and the relative contribution of host and pathogen factors in susceptibility to infective endocarditis has not been determined. It is important to gain an improved understanding of the host-bacterial interactions that contribute to disease outcome as this could facilitate the development of novel therapeutic approaches to the treatment of *S. aureus* infective endocarditis.

1.9 Aims

This research aims to investigate the contribution of host and pathogen factors to the development of infective endocarditis, with a view to identifying novel targets for future therapeutics. The aims are as follows:

1. Examination of the capacity of *S. aureus* clinical isolates to promote platelet aggregation after growth in human blood.
2. Investigation into the association of platelet activation with susceptibility to infective endocarditis.
3. Determination of the influence of platelet receptor polymorphisms on *S. aureus*-induced platelet aggregation and clinical outcome in infective endocarditis.

CHAPTER 2

GENERAL MATERIALS AND METHODS

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2.1 Subjects

One hundred and ninety-four healthy volunteers with no evidence of clinically significant co-existing conditions, recent infection or drug therapy, were recruited in Edinburgh between July 2007 and April 2009. Forty-four patients admitted to the Edinburgh Royal Infirmary (ERI) with a diagnosis of infective endocarditis fulfilling the modified Duke criteria (Table 1.2) (Li et al., 2000b), were recruited between November 2006 and March 2009. Four patients with *S. aureus* bacteraemia, defined as blood culture positivity for *S. aureus* not fulfilling the modified Duke criteria, were recruited over the same time period.

Exclusion criteria for all subjects included age less than 18 years and the presence of acute or chronic confusional states that precluded capacity to provide consent. Written informed consent was obtained from all participants. All studies were conducted following approval from the Lothian National Health Service (NHS) Research Ethics Committee.

2.2 Clinical outcomes and definitions

Data for infective endocarditis patients were collected prospectively for the index presentation and a 3-month period thereafter. The diagnosis of embolism was based on non-invasive radiological investigations and determined from the patient's history and clinical notes. Cutaneous manifestations were excluded, as their aetiology is considered multifactorial (Bashore *et al.*, 2006). Systematic computed tomography surveillance was not performed.

Relapse was defined as a new episode of infective endocarditis caused by a microorganism of the same genus and species, while a recurrence was defined as a new episode of infective endocarditis caused by a different microorganism (Mansur *et al.*, 2001). The composite clinical end-point was defined as the presence of embolism, heart failure, need for surgery or death in patients with infective

endocarditis.

2.3 Echocardiography

Images were acquired in all patients from transthoracic and/or transoesophageal echocardiogram windows performed early in the acute phase of the infective endocarditis process using 2-dimensional transducers capable of second and ultra-harmonic imaging, attached to either Vingmed (GE Healthcare, Bedford, UK) or iE33 (Philips, Surrey, UK) platforms. All data were stored digitally. Transthoracic and transoesophageal echocardiography images for determination of vegetation size and mobility were reviewed on EchoPAC v 7.0.1 for the PC (GE Healthcare Vingmed Ultrasound, Bedford, UK) by two independent experienced observers who were blinded to patient history, clinical course, platelet activation and platelet receptor genotype.

Vegetations were identified as distinctly abnormal echogenic masses attached to the endothelial surface of valves or endocardium. The largest length and width obtainable in any one frame throughout the cardiac cycle was measured for the largest vegetation if more than one vegetation was present (De Castro *et al.*, 1997). The mobility of vegetations was classified as previously described: absent, fixed vegetation with no detectable independent motion; low, vegetation fixed at the base but with a mobile free edge; moderate, pedunculated vegetation that remains within the same chamber throughout the cardiac cycle; severe, prolapsing vegetation that crosses the coaptation plane of the leaflets during the cardiac cycle (Di Salvo *et al.*, 2001).

2.4 Blood sampling

Venepuncture was performed using a 19-gauge (1.1 mm) needle or a 7 French (2.3 mm) triple-lumen central venous catheter into a 30 ml syringe. Care was taken to

ensure a smooth blood draw with minimal trauma. Three ml blood was transferred into a tube containing the direct thrombin inhibitor D-phenylalanyl-L-propyl-L-arginine chloromethylketone (PPACK; Haematologic Technologies Inc, Vermont, USA) at a final concentration of 75 μM for determination of platelet activation, and 9 ml into a tube containing 1.6 mg/ml of ethylenediamine tetraacetic acid (EDTA) to obtain plasma for platelet receptor polymorphism genotyping. Plasma was stored at -20°C until use.

Blood samples for isolation of platelet-rich plasma (PRP) for turbidimetric platelet aggregometry and flow cytometry were taken from a subset of healthy volunteers into 50 ml syringes containing 0.38% (w/v) sodium citrate. For isolation of washed platelets, blood was drawn into 50 ml syringes containing 15% (w/v) acid-citrate dextrose (7.32 g/l citric acid anhydrous, 22 g/l Na citrate, 24.52 g/l dextrose). For use as a growth medium, blood was sampled using a 19-gauge needle into a 60 ml syringe containing 5 international units (IU)/ml of unfractionated heparin (Roche, Welwyn Garden City, UK).

2.5 Platelet receptor polymorphism genotyping

Human genomic DNA was extracted from plasma using the bioMerieux NucliSens easyMag (bioMerieux, Basingstoke, UK) according to the manufacturer's instructions, and adjusted to a final concentration of 10 ng/μl in deionised water.

GPIIIa $Pl^{A1/A2}$, FcγRIIa H131R, GPIb HPA-2 and Kozak sequence polymorphisms were determined using the ABI 7900HT Sequence Detection System (Applied Biosystems, Warrington, UK). Primers 5' CTGAAAGGCAATGAGCTGAAGAC 3' and 5' TTGTTAGCCAGACTGAGCTTCTC 3', were used to amplify the polymorphic site of HPA-2 (Higgins *et al.*, 2004), while other polymorphisms were determined with proprietary oligonucleotide sequences and TaqMan® probes (Applied Biosystems, Warrington, UK). GPIb VNTR alleles were determined by PCR with a Whatman Biometra Tgradient PCR thermal cycler (Thistle Scientific,

Glasgow, UK). PCR reactions included 200 nM each of forward primer 5' ACACTTCACATGGAATCCAT 3' and reverse primer 5' GGGTCATTTCTGGAGCTCTC 3' (Ishida *et al.*, 1995), 1 U Taq polymerase, 200 μM deoxynucleotide triphosphate (dNTPs) and 10 ng genomic DNA in buffer (Promega, Southampton, UK). Thermocycling parameters included a 5 min initial denaturing step at 95°C, followed by 35 cycles consisting of 1 min at 95°C (denaturing), 1 min at 58°C (annealing) and 2 min at 72°C (extension). PCR products were resolved by electrophoresis in 2% (w/v) agarose.

2.6 Generation of platelet-rich plasma (PRP) and washed platelets

Citrated blood was centrifuged at 150 × *g* for 10 min with a bench-top 5810R Centrifuge (Eppendorf, Cambridge, UK) to generate PRP. The PRP fraction was removed and an aliquot was further centrifuged at 1500 × *g* for 10 min to generate platelet-poor plasma (PPP). Autologous PPP was used to standardise PRP to a platelet count of 200 × 10^9/l after the platelet count was determined on a Coulter® A.T™ series analyser (Beckman Coulter Inc., High Wycombe, UK).

For preparation of washed platelets, PRP was generated from blood anticoagulated with acid-citrate dextrose and adjusted to a pH of 6.5 with acid-citrate dextrose, prior to addition of 1 μM prostaglandin E_1 and 1 U/ml apyrase (both from Sigma, Dorset, UK). Samples were centrifuged at 720 × *g* for 10 min to obtain a platelet pellet, which was gently resuspended in modified HEPES-Tyrode's (JNL) buffer (6 mM dextrose, 130 mM NaCl, 9 mM $NaHCO_3$, 10 mM Na citrate, 10 mM Tris-base, 3 mM KCl, 0.8 mM KH_2PO_4, 0.9 mM $MgCl_2$; pH 7.4) (O'Brien et al., 2002a). Washed platelet suspensions were standardised to a platelet count of 200 × 10^9/l in JNL buffer with addition of 1 mg/ml human fibrinogen (Calbiochem, Nottingham, UK) and 2 mM Ca^{2+}. All platelet preparations were used within 2.5 h of venepuncture.

2.7 Bacterial strains

A total of 27 clinical and laboratory *S. aureus* isolates were used for *in vitro* experiments, including 5 isolates from patients with infective endocarditis, 6 from subjects with asymptomatic nasal colonisation, 5 bacteraemia strains and an array of laboratory and deletion mutant strains (Table 2.1). The infective endocarditis and commensal strains were obtained from Oxfordshire, United Kingdom between 1997 and 1998 and kindly gifted by Dr. Nicholas P. Day (Day *et al.*, 2001). *S. aureus* strains Newman, P1, 8325-4 and associated FnBPA and FnBPB knockouts (DU5998 and DU5883, respectively) were donated by Professor Timothy J. Foster (Moyne Institute of Preventative Medicine, Trinity College, Dublin, Ireland). *S. aureus* strain 132 and derivative mutants were kindly gifted by Iñigo Lasa (Instituto de Agrobiotecnología, Universidad Pública de Navarra-CSIC, Pamplona, Spain). The bacteraemia strains were obtained from patients admitted to the ERI from March to July 2007 with positive blood cultures for *S. aureus* and no evidence of infective endocarditis on transthoracic echocardiography. The Scottish MRSA Reference Laboratory, Glasgow, UK, performed MLST of the bacteraemia strains. All *S. aureus* strains were methicillin-sensitive apart from strain 132 and its derivative mutants.

Table 2.1. *S. aureus* strains used for *in vitro* experiments.

Strain	Characteristics	ST	Reference
126	Infective endocarditis	8	(Feil *et al.*, 2003)
206	Infective endocarditis	25	(Feil *et al.*, 2003)
207	Infective endocarditis	15	(Feil *et al.*, 2003)
209	Infective endocarditis	30	(Feil *et al.*, 2003)
383	Infective endocarditis	22	(Feil *et al.*, 2003)
3005	Commensal	15	(Feil *et al.*, 2003)
3010	Commensal	15	(Feil *et al.*, 2003)
3050	Commensal	30	(Feil *et al.*, 2003)
3081	Commensal	76	(Feil *et al.*, 2003)
3117	Commensal	30	(Feil *et al.*, 2003)
3125	Commensal	134	(Feil *et al.*, 2003)
SAB001	Bacteraemia	5	Current study
SAB002	Bacteraemia	3	Current study
SAB003	Bacteraemia	217	Current study
SAB004	Bacteraemia	30	Current study
SAB005	Bacteraemia	487	Current study
Newman	Wild type laboratory strain	8	(Duthie & Lorenz, 1952)
P1	Wild type. Rabbit virulent strain	973	(Sherertz *et al.*, 1993)
P1Δfnb (*fnbA*::Tc^r, *fnbB*::Em^r, DU5998)	FnBPA and FnBPB knockout of strain P1	973	(Roche *et al.*, 2004)

Strain	Characteristics	ST	Reference
DU5998 pFNBA4 (*fnbA*::Tc^r, *fnbB*::Em^r, Pfnba4:*fnbA*$^+$ Cm^r)	DU5998 complemented with plasmid expressing FnBPA	973	(Fitzgerald et al., 2006b)
DU5998 pFNBB4 (*fnbA*::Tc^r, *fnbB*::Em^r, Pfnba4:*fnbB*$^+$ Cm^r)	DU5998 complemented with plasmid expressing FnBPB	973	(Fitzgerald et al., 2006b)
P1 *clfA5, clfB::lacZ*[Em^r] Δ*sdrCDE*::Tc^r *spa*::Ka^r (DU6011)	ClfA, ClfB, Sdr C, Sdr D, Sdr E and SpA knockout of P1	973	(Fitzgerald et al., 2006b)
8325-4	NCTC 8325 cured of prophages	8	(Novick, 1967)
8325-4Δfnb (8325-4 *fnbA*::Tc^r, *fnbB*::Em^r, DU5883)	FnBPA and FnBPB knockout of strain 8325-4	8	(Greene *et al.*, 1995)
132	Wild type clinical isolate; MRSA	ND	(Vergara-Irigaray *et al.*, 2009)
132ΔIsdA (sasE::pMAD Em^r)	Insertional IsdA mutant of 132	ND	(Vergara-Irigaray *et al.*, 2009)
132ΔIsdB (sasJ::pMAD Em^r)	Insertional IsdB mutant of 132	ND	(Vergara-Irigaray *et al.*, 2009)

ST, sequence type by multi-locus sequence typing; Tc^r, tetracycline-resistant; Em^r, erythromycin-resistant; Cm^r, chloramphenicol-resistant; Ka^r, kanamycin-resistant; MRSA, methicillin-resistant *S. aureus*; ND, not determined.

2.8 Bacterial culturing

S. aureus strains were grown on tryptic soy agar (TSA) plates (Oxoid, Basingstoke, UK) statically at 37°C and grown in liquid media as outlined below. The following antibiotics were incorporated into the media where appropriate (erythromycin 5 or 10 μg/ml, chloramphenicol 10 μg/ml).

2.8.1 Bacterial growth in nutrient broth

Single colonies of *S. aureus* strains were used to inoculate 25 ml of brain-heart infusion (BHI) (Oxoid, Basingstoke, UK) and incubated overnight at 37°C with constant rotation at 200 rpm, to reach the stationary phase of growth. A 1:200 dilution of overnight culture was made in 25 ml of BHI, with incubation under the same conditions as above and grown to mid-exponential phase (approximately 1.5 h to 3 h as determined from growth curves). Bacterial growth was assessed by measurement of the optical density at 600 nm (OD_{600}) using a spectrophotometer (Cecil Aurius CE2021, Thistle Scientific Ltd., Glasgow, UK).

2.8.2 Bacterial growth in whole human blood

Single colonies of *S. aureus* isolates were inoculated in 5 ml of heparinised whole human blood at 37°C with shaking at 200 rpm in an orbital shaker, until they reached the stationary phase of growth. Stationary phase cultures were subcultured into 40 ml of heparinised whole human blood at 10^6 CFU/ml and grown to either mid-exponential or stationary phase, as determined from growth curves, for use in *in vitro* experiments. Bacterial growth was determined by performing viable counts.

2.9 Processing of bacterial cultures for *in vitro* studies

S. aureus strains were grown to stationary and mid-exponential phase in BHI and blood. Bacteria were isolated from blood cultures as previously described for Group A *Streptococcus,* with modifications (Graham *et al.*, 2005). Briefly, blood cultures were resuspended in 5 volumes of erythrocyte lysis (EL) buffer (Qiagen, Crawley, UK) and incubated for 20 min on ice. Following 10 min centrifugation at 4000 rpm at 4°C, the supernatant was discarded and the cells were washed with 2 volumes of EL buffer. Cells were washed once in 100 U/ml of unfractionated heparin and 10 mM calcium chloride solution to prevent cell clumping (O'Connell *et al.*, 1998, Shin *et al.*, 2005), and twice in phosphate-buffered saline (PBS) at 5000 × *g* for 3 min. Cultures were resuspended in PBS to an OD_{600} value corresponding to a viable count of 2×10^9 CFU/ml for use in platelet aggregometry.

For bacteria grown in BHI, cultures were centrifuged at 5000 × *g* for 3 min, washed twice, and resuspended in 1 ml of PBS to an OD_{600} of 1.6, corresponding to approximately 2×10^9 CFU/ml, for platelet aggregometry and activation studies, and an OD_{600} of 1.0 for fibronectin adherence assays (Fitzgerald et al., 2006b). Culture volumes of 1ml were processed at the stationary phase of growth and 25 ml at the exponential growth phase.

To process bacteria for *S. aureus*-platelet adhesion studies, stationary and exponential phase BHI cultures were washed twice in cold 0.05 M Tris-HCl buffer containing 0.1 M NaCl and 0.02 M EDTA, pH 7.25 (Yeaman et al., 1992c). Bacteria were labelled with 100 μg/ml of Hoechst 33342 (Sigma, Dorset, UK) prior to incubation in the dark for 2 h at 4°C, followed by twice washing in Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.2 mM Na_2HPO_4, 12 mM $NaHCO_3$, 5.5 mM dextrose, pH 7.4) and resuspension to an OD_{600} of 1.6.

2.10 Flow cytometry

2.10.1 *S. aureus*-platelet adhesion

S. aureus-platelet adhesion was determined by incubation of 9×10^7 Hoechst 33342-labelled bacterial cells with 9×10^6 platelets in PRP at room temperature for 5 min, followed by labelling with 1% fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD42a antibody (Serotec, Kidlington, UK) for 30 min and fixing with 1 ml 1% (v/v) paraformaldehyde. Fluorescent data were determined within 24 h of fixation using a FACSVantage SE cell sorter (Becton Dickinson, Oxford, UK) with FACSDiVa software (Becton Dickinson, Oxford, UK). One hundred thousand platelets were collected for each sample and distilled water, flow buffer (Becton Dickinson, Oxford, UK) and 0.05 M Tris-HCl buffer containing 0.1 M NaCl and 0.02 M EDTA, pH 7.25, served as negative controls. *S. aureus* bound to platelets were identified as cells positive for both Hoechst and FITC fluorescent signals, and data were analysed using FlowJo v 8.8.6 (Tree Star Inc., Oregon, USA) to determine the percentage of platelets bound to *S. aureus* and the percentage of *S. aureus* bound to platelets.

2.10.2 Baseline and agonist-induced platelet activation

Within 5 min of transfer into a PPACK tube, blood was labelled at room temperature with the appropriate monoclonal antibodies for determination of baseline platelet activation, as previously described (Sarma *et al.*, 2002, Gudmundsdottir *et al.*, 2006). Briefly, mouse anti-human CD14-phycoerythrin (PE) (Inverness Medical, Stockport, UK) and anti-CD42a-FITC were added to 60 µl of blood at a 1:40 dilution for detection of PMA. Anti-CD42a-FITC and mouse anti-human CD62P-PE (Serotec, Kidlington, UK) at a 1:2 dilution were used to label 5 µl of blood for detection of platelet P-selectin expression. The appropriate isotope mouse IgG_1 antibodies (Serotec, Kidlington, UK) and flow buffer served as negative controls. Following 20 min of immunolabelling, PMA and P-selectin samples were fixed with 500 µl FACS

Lyse solution (Becton Dickinson, Oxford, UK) and 1.425 ml 1% (v/v) paraformaldehyde, respectively.

Platelet activation was quantified within 24 h of sample fixation using dual-channel flow cytometry on the FACSCalibur (Becton Dickonson, Oxford, UK), with at least 2,000 monocytes and 7,500 platelets collected for each sample. PMAs were defined as monocytes expressing CD14 that were also positive for CD42a and P selectin-expressing platelets were defined as CD42a-expressing platelets that were positive for CD62P. Data were acquired and analysed using CellQuest software (Becton Dickonson, Oxford, UK), and results expressed as a percentage of the monocyte and platelet cell population, respectively.

For determination of agonist-induced platelet activation, bacterial suspensions were added to PRP at a ratio of 10:1, incubated for 30 min at room temperature, followed by labelling with 2% anti-CD42a-FITC, anti-CD62P-PE or the appropriate IgG_1 isotype control for 30 min. Samples were fixed with 1 ml 1% (v/v) paraformaldehyde and platelet P-selectin expression determined as described above using a FACScan flow cytometer (Becton Dickinson, Oxford, UK). The pharmacological protease-activated receptor-1 agonist SFLLRN-NH_2 (1 μM; Clinalfa, Laufelfingen, Switzerland) was used as a positive control. Baseline platelet P-selectin expression was also determined using equivalent volumes of vehicle alone (PBS and 0.9% NaCl instead of bacterial and pharmacological agonists, respectively), in order to calculate relative platelet activation (see Section 5.3.3).

2.11 Platelet aggregometry

Platelet aggregometry was performed as described previously (Fitzgerald et al., 2006b). Briefly, 5×10^7 bacterial cells (in 25 μl PBS) were added to 4.5×10^7 platelets (in 225 μl PRP or washed platelets) in siliconised glass cuvettes with constant stirring at 37°C. Platelet aggregation was assayed by light transmission in a PAP-4 aggregometer (Bio-Data, Alpha Laboratories, Eastleigh, UK) in triplicate.

PPP and JNL were used as the 100% light transmission references for PRP and washed platelets, respectively. *S. aureus* strain Newman at either the stationary or exponential phase of growth, SFLLRN-NH_2 (0.1 μM) ADP (5 μM; Trinity Biotech, Wicklow, Ireland) and ristocetin (0.1 μM; American Biochemical and Pharmaceutical, London, UK) were used as agonist positive controls. The lag time to platelet aggregation was measured as the time from addition of agonist to the initiation of platelet aggregation up to a 25 min limit. The maximal percentage platelet aggregation and rate of aggregation were also determined (Figure 2.1).

2.12 Fibrinogen-binding assays

Adherence of bacteria to immobilised fibrinogen was performed as previously described (Hartford *et al.*, 1997). Briefly, 96-well flat-bottomed plates (Nunc, Loughborough, UK) were coated with serial dilutions of 100 μl of 0 to 50 μg/ml human fibrinogen in PBS and incubated at 4°C overnight. Plates were thrice washed with 100 μl PBS, and then incubated with 100 μl of 2 mg/ml filter-sterilised bovine serum albumin (Sigma, Dorset, UK) for 1 h at 37°C. Following three washes with PBS, 1.25 x 10^8 *S. aureus* cells were added to each well and incubated for 2 h at 37°C under constant rotation, followed by removal of non-adherent cells by thrice washing with 100 μl PBS. Cells were fixed with 100 μl 25% (v/v) formaldehyde (VWR, Lutterworth, UK) for 30 min at room temperature, stained with 100 μl 0.5% (w/v) crystal violet (Sigma, Dorset, UK) for 1 min, and solubilised with 100 μl 10% (v/v) acetic acid (Fisher Scientific, Loughborough, UK). Plates were washed three times with 100 μl PBS between each fixing or staining stage.

The absorbance at 590 nm (A_{590}) was determined in an enzyme-linked immunosorbent assay (ELISA) plate reader (VERSAmax microplate reader, Molecular Devices, Wokingham). *S. aureus* strain Newman at the stationary phase of growth was used as a positive control, while PBS served as a negative control at fibrinogen concentrations of 50 μg/ml. Experiments were performed in triplicate.

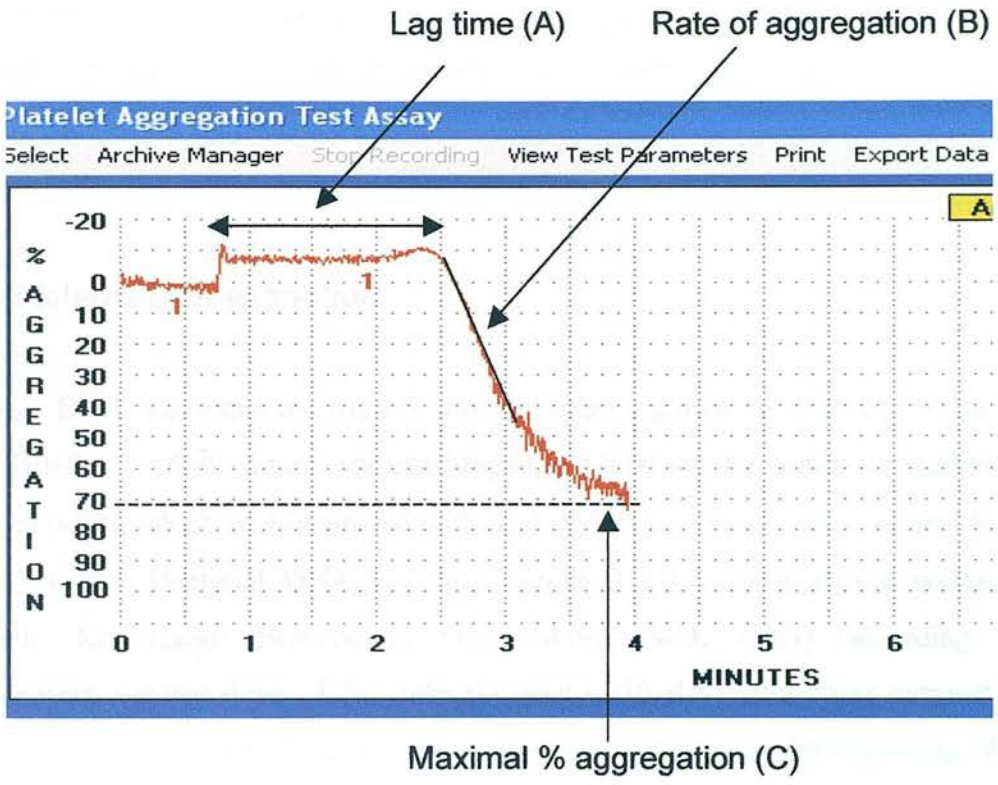

Figure 2.1. Measures of platelet aggregation. Lag time was taken as the time taken from addition of agonist to initiation of platelet aggregation (A), rate of aggregation as the gradient during the steepest part of the aggregation curve (B) and maximal percentage aggregation as the level of percentage platelet aggregation at which the tracing plateaued (C).

A_{590} readings obtained for the negative control were subtracted for values obtained for each strain at each fibrinogen concentration to generate a curve with plateaued fibrinogen binding. A_{590} readings at saturation of fibrinogen binding (fibrinogen concentration of 12.5 μg/ml) were used for analyses.

2.13 Bacterial DNA extraction

Genomic DNA was extracted from *S. aureus* cultures grown to stationary phase in BHI. Briefly, 1 ml of culture was centrifuged at 14000 × *g* for 6 min to generate a bacterial pellet, which was incubated with 5 μl of 5 mg/ml lysostaphin (Ambi, New York, USA). Bacterial DNA was then extracted using the PurElute Bacterial Genomic Kit (Edge Biosystems, Gaithersburg, MD, USA) according to manufacturer's instructions. DNA integrity was verified by subjecting samples to electrophoresis in 1% (w/v) agarose, and yield quantified using the Nanodrop ND-1000 spectrophotometer (Fisher Scientific, Loughborough, UK). Bacterial DNA samples were standardised to a concentration of 350 ng/μl in deionised water and stored at 4°C until use.

2.14 Bacterial RNA extraction and conversion to complementary DNA (cDNA)

For isolation of bacteria for RNA extraction, BHI and blood cultures grown to stationary phase were pre-treated with RNA protect (Qiagen, Crawley, UK) according to manufacturer's instructions, processed as described in Section 2.9, and washed for 10 min at 5000 × *g* in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE buffer, Fluka, Dorset, UK). *S. aureus* cells were then resuspended in 500 μl TE buffer with 100 μg/ml lysostaphin, followed by incubation at 37°C for 30 min. Bacterial RNA was extracted using the RNeasy mini kit (Qiagen, Crawley, UK) according to manufacturer's instructions.

Removal of contaminating DNA was performed using the Ambion TURBO DNA-free kit (Applied Biosystems, Warrington, UK) as per manufacturer's instructions. Samples were then centrifuged at 8000 × *g* for 2 min and 25 µl of supernatant retained as DNA-free RNA. The absence of contaminating DNA was verified by negative results when performing PCR on RNA samples with a Whatman Biometra Tgradient PCR thermal cycler. PCR reactions included 300 nM each of forward and reverse *16S rRNA* primers (Table 2.2), 1 U Taq polymerase, 200 µM dNTPs and 50 ng RNA in buffer (Promega, Southampton, UK). Thermocycling parameters included a 3 min initial denaturing step at 94°C, followed by 30 cycles consisting of 1 min at 94°C (denaturing), 1 min at 60°C (annealing) and 90 s at 72°C (extension) with a further 10 min at 72°C (final extension). Positive controls for all PCR reactions comprised of genomic DNA extracted from *S. aureus* strain Newman, while DNA- and RNA-free water served as a negative control. PCR products were resolved by electrophoresis in 2% (w/v) agarose.

RNA concentration and quality were determined using the Nanodrop ND-1000 spectrophotometer, which quantifies nucleic acid yield and A_{260}:A_{280} ratios (the latter should be approximately 1.8 to 2.0), and by examination of the integrity of RNA samples after electrophoresis in a 2% (w/v) agarose gel. Bacterial RNA samples were stored at -70°C until use.

RNA (50 ng) was converted to complementary DNA (cDNA) using the AffinityScript QPCR cDNA synthesis kit (Agilent, Stockport, UK) according to manufacturer's instructions.

2.15 Quantitative Reverse-Transcriptase PCR (qRT-PCR)

Quantitative reverse-transcriptase PCR (qRT-PCR) was performed using the MX3000P quantitative-PCR instrument (Stratagene, Stockport, UK), with the SYBR Green Supermix-UDG kit (Invitrogen, Paisley, UK) according to manufacturer's instructions, 300 nM each of forward and reverse primers for *clfA* and *16S rRNA*

(Table 2.2) and 100 ng cDNA prepared from *S. aureus* stationary phase BHI and blood cultures. qRT-PCR cycling conditions consisted of a 10 min initial denaturing step at 95°C, followed by 40 cycles consisting of 20 s at 94°C (denaturing), 20 s at 60°C (annealing) and 20 s at 72°C (extension), to generate PCR products of 115 bp (*16S rRNA*) and 276 bp (*clfA*).

Positive controls included 10-fold serial dilutions of genomic DNA extracted from *S. aureus* strain Newman, which were used to generate standard curves of template concentration against threshold cycle (C_t, the cycle at which fluorescence is determined to be significantly greater than background signal), for both *16S rRNA* and *clfA* primers on the MxPro QPCR v. 3.00 software (Stratagene, Stockport, UK). Gradients of and R^2 values derived from standard curves were used to verify primer-binding efficiency. Negative controls included RNA treated with the AffinityScript QPCR cDNA synthesis kit in the absence of reverse transcriptase (no-RT) and wells containing DNA- and RNA-free water rather than cDNA (non-template controls or NTCs).

qRT-PCR product integrity and absence of contamination in negative controls were verified by analysis of amplicon melting temperatures in dissociation curves generated by the MxPro QPCR v. 3.00 software, and by subjecting qRT-PCR products to electrophoresis on a 2% (w/v) agarose gel. qRT-PCR-transcript levels of *clfA* relative to the normalising gene *16s rRNA* were determined using the equation

$$\frac{(1+E_{clfA})^{-C_{tclfA}}}{(1+E_{16S})^{-C_{t16S}}}$$

where E_{clfA} and E_{16S} represent the efficiency of amplification of *clfA* and *16S rRNA* respectively (value between 0 and 1), and C_{tclfA} and C_{t16S} represent the threshold cycles for *clfA* and *16S rRNA,* respectively.

Table 2.2. Oligonucleotide primers used for quantitative reverse-transcriptase PCR.

Primer name	Oligonucleotide sequence	Product size (bp)	Reference
ClfU	5′ GGCGTGGCTTCAGTGCTTGTA 3′	276	(Wolz *et al.*, 2002)
ClfL	5′ CACCAGTTACCGGCGTTTCTTC 3′		
16SF	5′ TATGGAGGAACACCAGTGGCGAAG 3′	115	(Ster *et al.*, 2005)
16SR	5′ TCATCGTTTACGGCGTGGACTACC 3′		

2.16 Statistical analyses

Data analysis was performed using SPSS v. 16.0 (SPSS Inc, Chicago, IL, USA) for the Macintosh. Values are reported as mean ± standard deviation and the normality of data distribution determined using the Shapiro-Wilk test.

The chi-squared test was used to compare polymorphism frequencies. Parametric data were analysed using the two-tailed Student t-test, analysis of variance (ANOVA) or General Linear Model, and non-parametric data analysed by the Kruskal-Wallis test with Mann-Whitney U correction. Linear regression was used to compare numerical data and multinomial logistic regression analysis was used to determine correlations between categorical variables. Statistical significance was taken as $P<0.05$ for univariate analyses and $P<0.10$ for multivariate analyses.

CHAPTER 3

EXAMINATION OF THE CAPACITY OF *S. AUREUS* CLINICAL ISOLATES TO PROMOTE PLATELET AGGREGATION AFTER GROWTH IN HUMAN BLOOD

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3.1 Introduction

MSCRAMMs, such as FnBPA, FnBPB, ClfA, ClfB and SpA contribute to *S. aureus*-induced platelet aggregation following bacterial growth in nutrient-rich media (Fitzgerald et al., 2006b, Loughman et al., 2005, Miajlovic et al., 2007, O'Brien et al., 2002a). However, experimental conditions employed in these studies do not reflect the *in vivo* milieu encountered by bacteria during growth in the human bloodstream, and *S. aureus* growth in human blood may influence MSCRAMM expression and functionality (Novick, 2003, Pluym *et al.*, 2008, Schlievert *et al.*, 2007, Ward *et al.*, 1996). In order to investigate this hypothesis, whole human blood was utilised as a bacterial growth medium *ex vivo*. The ability of selected *S. aureus* clinical isolates to induce platelet aggregation after growth in human blood was compared to results obtained following bacterial growth in BHI.

3.2 Methods

S. aureus strains Newman, 383 and 3081 were grown in BHI and heparinised human blood as described in Section 2.8 in order to generate growth curves for *S. aureus* in each medium. Growth curves were used to determine the timing of mid-exponential and stationary phases of growth for *S. aureus* in each medium. Strains were grown in BHI for 8.5 h and OD_{600} readings taken every 30 to 60 min, while strains grown in human blood were cultured for 36 h, with viable counts determined every 2 h following the initial lag phase.

A selection of *S. aureus* clinical isolates (Table 2.1) were then grown to stationary and mid-exponential phases in blood and BHI, processed as described in Section 2.9 and used to stimulate aggregation of platelets in PRP as described in Section 2.11. The lag time and percentage platelet aggregation was determined for each agonist, with strain Newman at both growth phases used as a positive control.

In order to determine the role of ClfA in bacterium-platelet interactions, fibrinogen-binding assays were performed as described in Sections 2.9 and 2.12 using *S. aureus* clinical isolates grown to stationary phase in human blood and BHI. In addition, *clfA* gene transcription levels were determined for a selection of *S. aureus* strains grown to stationary phase in BHI and blood using qRT-PCR as described in Sections 2.14 and 2.15. The role of other selected MSCRAMMs in platelet aggregation induced by *S. aureus* was examined by performing platelet aggregometry using wild type, deletion mutant and complemented strains (listed in Table 2.1) grown to stationary phase in human blood. Strain Newman grown to stationary phase in BHI was used as a positive control for these experiments.

3.3 Results

3.3.1 *S. aureus* grows in whole human blood

In order to compare the growth profiles of *S. aureus* in human blood and BHI, growth curves were performed for 3 selected *S. aureus* strains (Newman, 383 and 3081) in each medium. When *S. aureus* was grown in blood, there was a lag phase of 10 h, with a mid-exponential phase at 14 h to 20 h and a stationary phase of growth from 32 h onwards (Figure 3.1A). Notably, strain Newman did not grow in blood. Bacterial counts at the stationary phase of growth varied from 7.61×10^8 to 5.54×10^9 CFU/ml. In contrast, the *S. aureus* growth curve in nutrient-rich BHI had a mid-exponential phase at 2 h to 2.5 h and reached stationary phase after 8 h of growth (Figure 3.1B), similar to previous findings (O'Brien et al., 2002a). The bacterial yield at stationary phase was equivalent to 1.77×10^{10} to 2.22×10^{10} CFU/ml, with corresponding OD_{600} values of 14.2 to 17.7, while the mid-exponential phase of growth was defined as an OD_{600} value of between 0.5 and 0.9. Doubling times for the selected strains averaged 30.7 min in BHI and 97.4 min in blood.

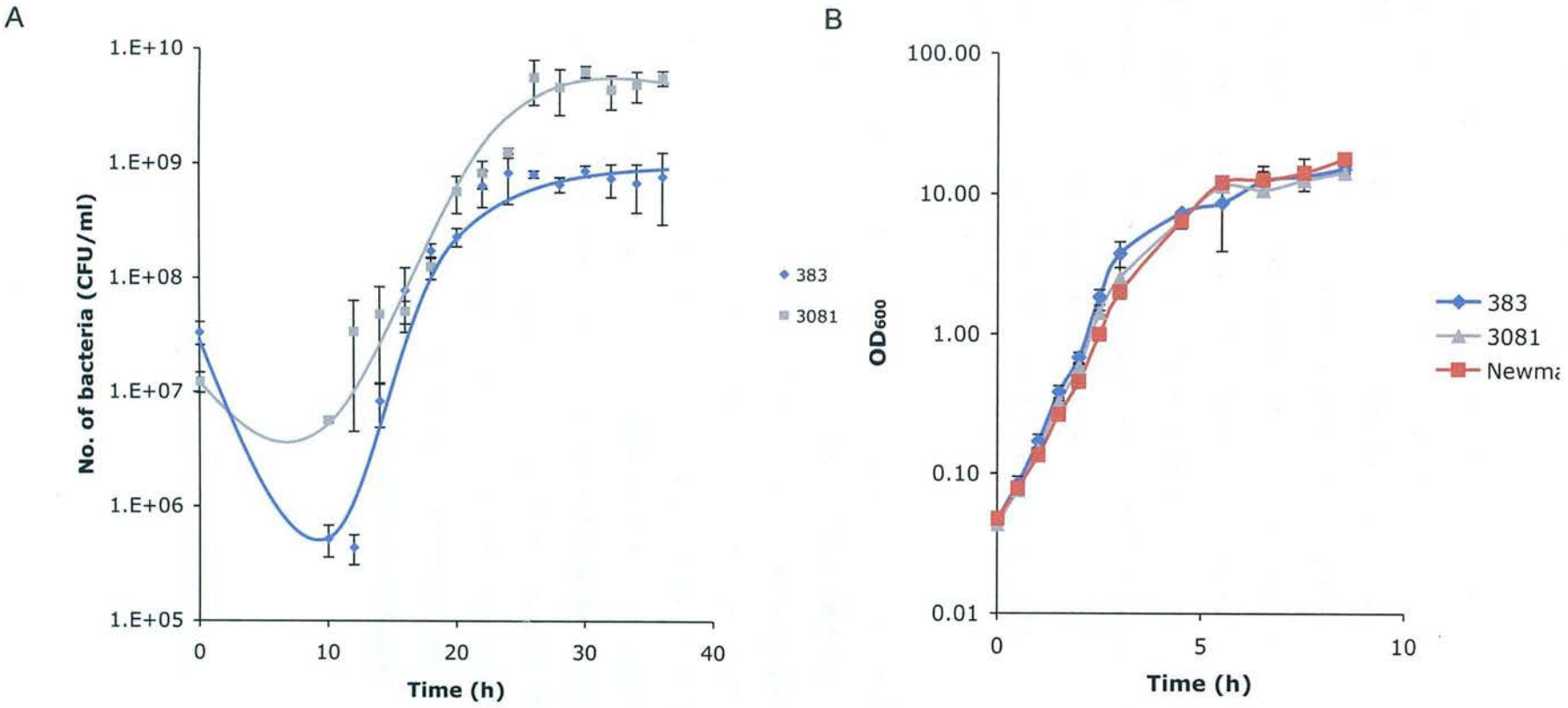

Figure 3.1. Growth curves for *S. aureus* infective endocarditis strain 383 (blue), nasal commensal strain 3081 (grey) and strain Newman (red) grown in human blood (A) and BHI (B). Strain Newman did not grow in blood. Bacterial counts for growth in blood (A) were determined by performing viable counts, while growth in BHI (B) was quantified by measuring the bacterial density using a spectrophotometer at OD_{600}.

3.3.2 Growth of *S. aureus* to stationary phase in blood confers a pro-aggregatory phenotype

S. aureus strains isolated from cases of infective endocarditis, nasal colonisation and bacteraemia and representing diverse STs (Table 2.1), were grown in both blood and BHI and used to induce platelet aggregation. There was strain-dependent variation in the ability of *S. aureus* to grow in blood (data not shown). However, all strains grown in blood were able to induce platelet aggregation at mid-exponential (Figure 3.2A) and stationary (Figure 3.2B) phases of growth, whereas 4 strains grown in BHI were unable to do so (P=0.096), indicating expression of bacterial surface components stimulating platelet aggregation by all strains grown in blood. Maximal percentage platelet aggregation could not be determined for strains grown in blood as residual erythrocytes were often present in washed cultures and would interfere with readings obtained using an optical aggregometer.

Considering that the protocol for processing *S. aureus* cultures differed depending on the growth media (see Section 2.9), it is feasible that additional washes employed for the isolation of *S. aureus* from blood cultures influenced the ability of strains to induce platelet aggregation. In order to rule out this possibility, a representative *S. aureus* strain (SAB002) was grown to stationary phase in BHI and subjected to 10 min preincubation in blood, washes with EL buffer, calcium chloride solution or heparin, either alone or in combination, representing the different processing stages (see Section 2.9). None of these processing steps influenced the ability of *S. aureus* to induce platelet aggregation (data not shown), suggesting that the observed variation in platelet aggregation following *S. aureus* growth in different media was due to differential expression of bacterial surface components.

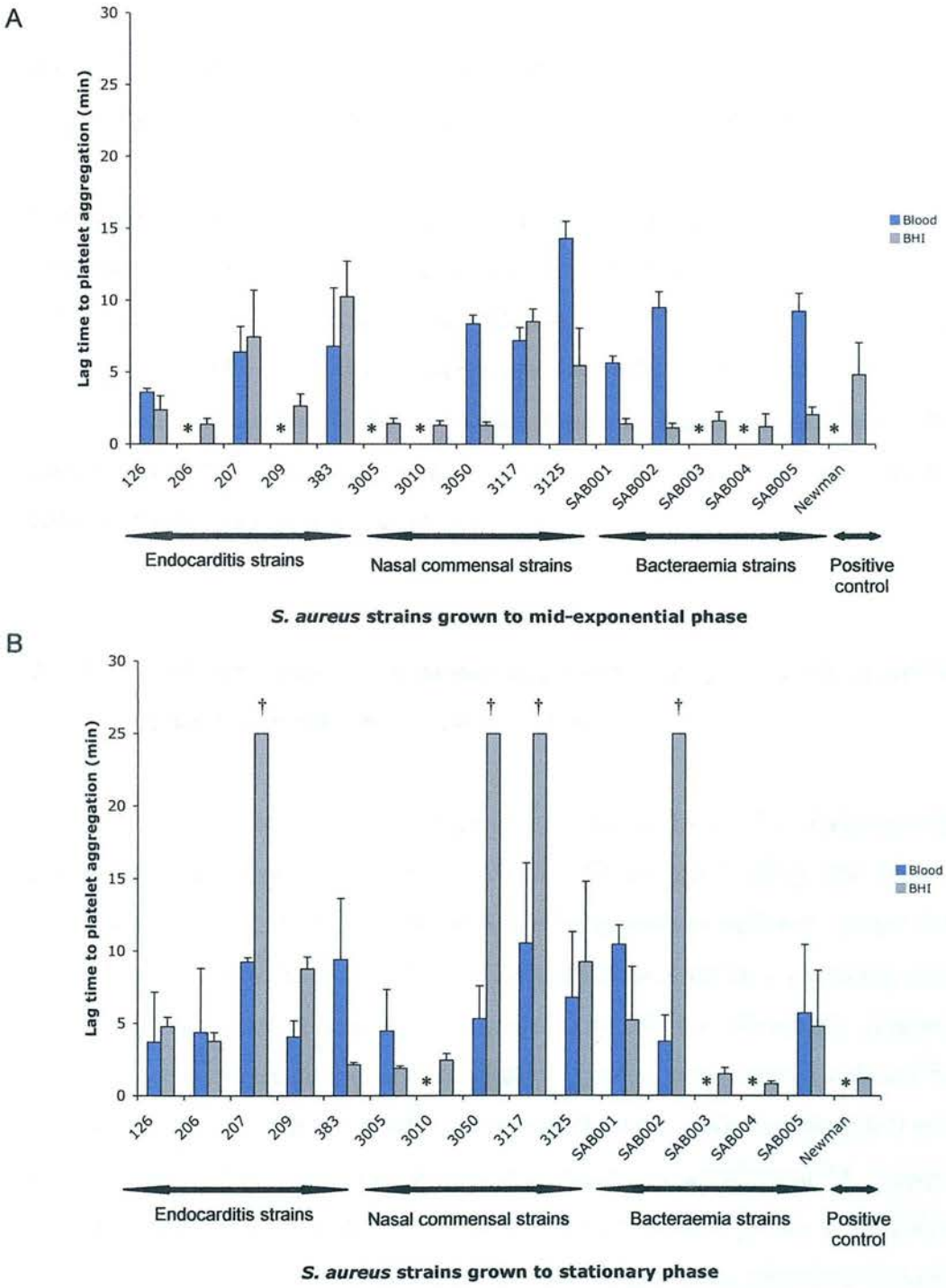

Figure 3.2. Lag time to platelet aggregation induced by *S. aureus* clinical isolates grown to mid-exponential (A) and stationary phase (B) in heparinised whole human blood (blue) and BHI (grey). Strain Newman grown in BHI served as a positive control. Experiments were performed in triplicate using three different platelet donors. * Unable to isolate sufficient bacterial cells for aggregometry † Absence of platelet aggregation at 25 min

3.3.3 Investigation into the bacterial components mediating platelet aggregation following *S. aureus* growth in human blood

The ability of some *S. aureus* strains to induce platelet aggregation after growth to stationary phase in blood but not BHI, indicates that bacterial components mediating platelet aggregation, such as MSCRAMMs, may be upregulated after bacterial growth in blood. In order to determine the role of MSCRAMMs in platelet aggregation induced by *S. aureus* strains grown in blood, plasma protein-binding assays, qRT-PCR and platelet aggregometry with wild type and MSCRAMM deletion mutant strains were performed.

3.3.3.1 *clfA* expression is downregulated during *S. aureus* growth to stationary phase in human blood

As ClfA binds platelets via fibrinogen (see Section 1.4.3.3.2), fibrinogen-binding assays were performed to ascertain whether fibrinogen binding and hence ClfA expression was upregulated following *S. aureus* growth to stationary phase in blood. For strains grown to stationary phase in BHI, all clinical isolates, including those that did not induce platelet aggregation, bound immobilised fibrinogen (Figure 3.3). However, strains that did not induce platelet aggregation exhibited reduced binding to 12.5 μg/ml fibrinogen (P=0.008). A representative *S. aureus* strain (207) grown in human blood did not bind immobilised fibrinogen, independent of EL, heparin and calcium washes (data not shown). The inability of cultures grown in *ex vivo* media to bind immobilised plasma proteins has previously been observed following *S. aureus* growth in peritoneal dialysate, and attributed to bacterial coating with plasma proteins during growth (Massey *et al.*, 2002). It is likely that the same phenomenon occurs during bacterial growth in human blood *ex vivo*.

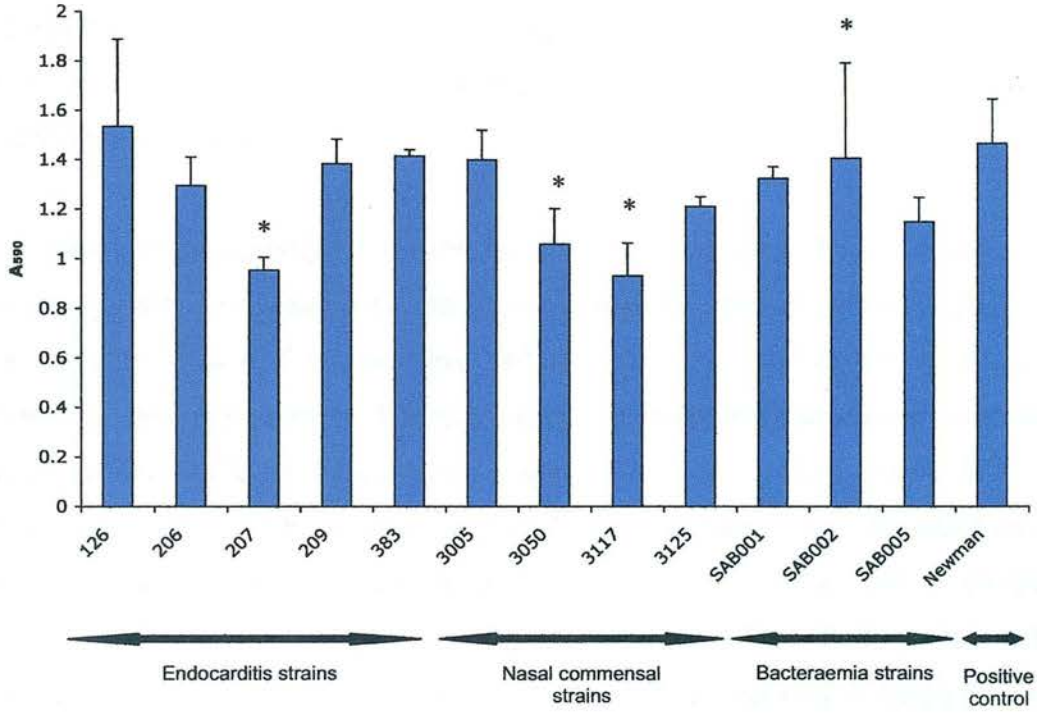

Figure 3.3. Binding of *S. aureus* clinical isolates grown to stationary phase in BHI to 12.5 μg/ml immobilised fibrinogen. Fibrinogen binding was determined as the absorbance at 590nm (A_{590}) for each bacterial agonist at a range of fibrinogen concentrations, but only data for 12.5 μg/ml fibrinogen were analysed. Strain Newman was used as a positive control. Experiments were performed in triplicate.
* Strains that did not induce platelet aggregation after growth to stationary phase in BHI.

Considering that ClfA is implicated in platelet aggregation induced by *S. aureus* cultures grown to stationary phase in nutrient broth (see Section 1.3.5.2), and that some strains grown to stationary phase in BHI which did not stimulate platelet aggregation had reduced fibrinogen binding, it was hypothesised that ClfA surface expression might be upregulated during growth in blood, conferring the ability to stimulate platelet aggregation.

Initial attempts to quantify ClfA expression on the *S. aureus* cell surface using flow cytometry were unsuccessful due to technical reasons (data not shown). qRT-PCR was therefore used to determine levels of *clfA* gene transcription during *S. aureus* growth to stationary phase in different media. Representative strains were selected, based on their ability to induce platelet aggregation after growth in both BHI and blood (strain 209) and blood only (strain SAB002) (Figure 3.4A). Standard curves for *clfA* and *16S rRNA* demonstrated efficient primer binding, and dissociation curves revealed that negative controls did not contain amplified PCR products (data not shown). Transcript levels for *16S rRNA* did not differ between cultures grown in blood and BHI (P=0.49 and P=0.42 for 209 and SAB002 respectively, data not shown), indicating that *16S rRNA* is an appropriate constitutively expressed control for qRT-PCR.

Unexpectedly, *clfA* transcription levels relative to the normalising gene *16S rRNA* were downregulated in blood as compared to BHI for both strains (Figure 3.4B). Further, there was no difference in lag time to platelet aggregation induced by the wild type *S. aureus* strain P1 and a derivative strain containing deletions of the genes encoding ClfA, ClfB, SdrCDE and SpA (DU6011) grown to stationary phase in blood (P=0.252, Figure 3.5).

Collectively, these data suggest that ClfA is unlikely to account for the increased platelet aggregation induced by *S. aureus* strains grown to stationary phase in blood.

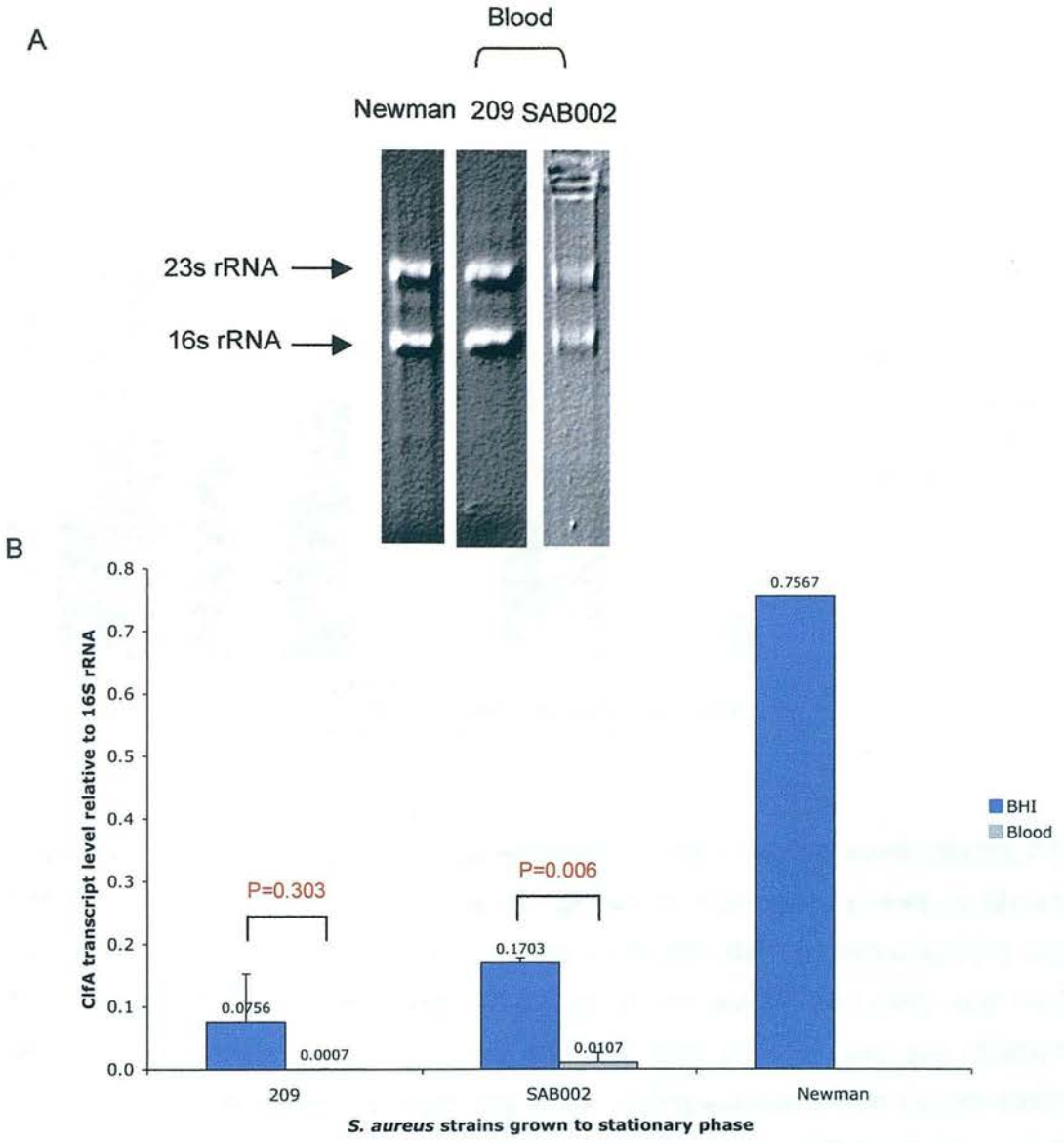

Figure 3.4. *clfA* expression during *S. aureus* growth in human blood. Electrophoresis of bacterial RNA extracted from *S. aureus* strain Newman grown in BHI and strains 209 and SAB002 grown in human blood on a 2% (w/v) agarose gel (A). Normalised *clfA* transcript levels for *S. aureus* strains 209 and SAB002 grown to stationary phase in BHI (blue) and heparinised human blood (grey) (B). Values are depicted above each bar. Strain Newman grown to stationary phase in BHI was used as a positive control. Experiments were performed in duplicate except for strain Newman, which was performed once.

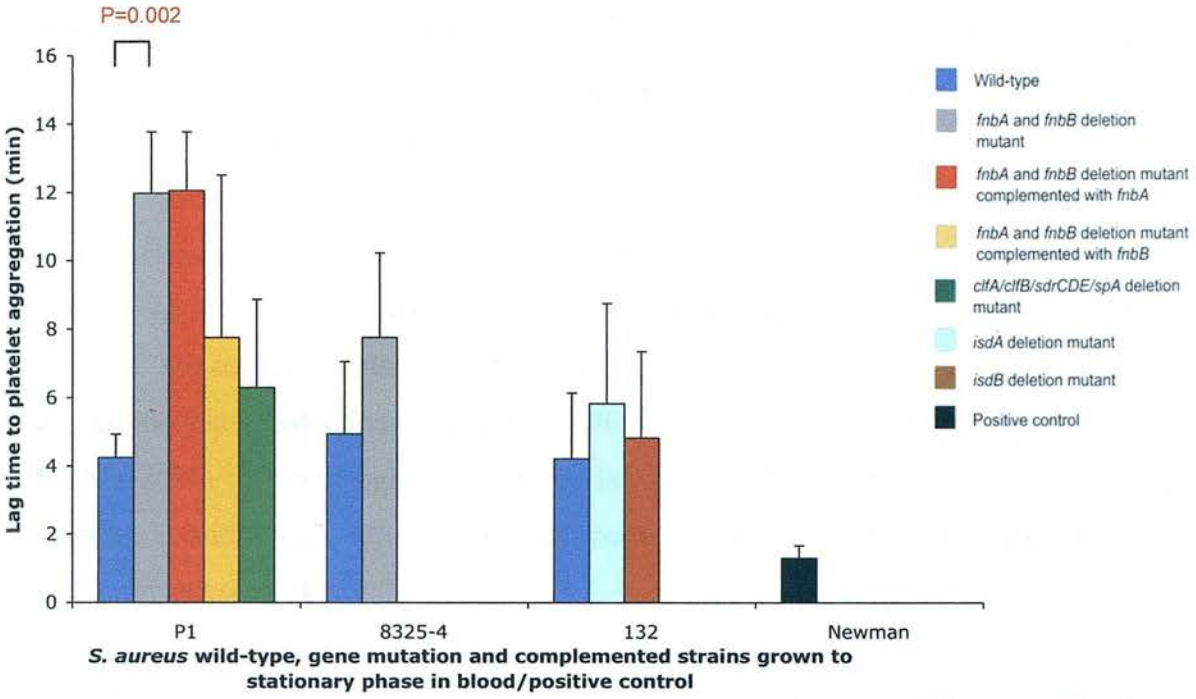

Figure 3.5. Lag time to platelet aggregation induced by *S. aureus* strains P1, 8325-4 and 132 and derivative mutants grown to stationary phase in blood. Lag times for wild type strains (dark blue), *fnbA* and *fnbB* deletion mutants of P1 and 8325-4 (DU5998 and DU5883 respectively, grey), the single *fnbA* (red) and *fnbB* (yellow) complements of DU5998 and the *clfA, clfB, sdrCDE* and *spa* deletion mutant of P1 (green) grown to stationary phase in heparinised whole human blood are depicted. Aggregometry was also performed using strain 132 (dark blue), *isdA* (light blue) and *isdB* (brown) single gene mutants. Strain Newman grown to stationary phase in BHI (black) served as a positive control for all experiments. Experiments were performed in triplicate using three different platelet donors.

3.3.3.2 Deficiency in IsdA and IsdB expression does not influence platelet aggregation induced by *S. aureus* strains grown to stationary phase in blood

Other selected bacterial surface components may influence platelet aggregation induced by *S. aureus* grown in blood. For example, Isd proteins bind to plasma proteins, are expressed after growth in human serum and contribute to disease in animal models of infection (Clarke *et al.*, 2004, Pishchany *et al.*, 2009, Wiltshire & Foster, 2001). Accordingly, the role of Isd proteins in platelet aggregation was examined using wild type, *isdA*- and *isdB*-deficient mutant strains grown to stationary phase in blood.

S. aureus strain 132 and its *isdA* and *isdB* single mutants grew in blood with no significant effect of either mutation on bacterial growth (data not shown). There was no difference in lag time to platelet aggregation induced by the *isdA* and *isdB* mutants in comparison to the wild type strain (P=0.163 for the *isdA* mutant and P=0.308 for the *isdB* mutant, Figure 3.5). Additionally, mutations of *clfB, sdrCDE* and *spA* genes in strain P1 did not influence the ability of *S. aureus* to induce platelet aggregation following growth in blood (P=0.252, Figure 3.5).

3.3.3.3 Deficiency in FnBPA and FnBPB expression increases lag time to platelet aggregation induced by *S. aureus* strains grown to stationary phase in blood

A previous study has demonstrated important roles for FnBPA and FnBPB in stimulation of platelet aggregation following *S. aureus* growth in nutrient broth (Fitzgerald *et al.*, 2006b). Here, the contribution of the fibronectin-binding proteins to platelet aggregation induced by *S. aureus* grown in blood was examined. Lag time was significantly increased with the double *fnb* mutant (DU5998) as compared to the wild type strain P1 (P=0.002, Figure 3.5), suggesting that either FnBPA or FnBPB

alone or in combination contribute to platelet aggregation induced by *S. aureus* grown to stationary phase in blood. The same trend was not observed with strain 8325-4 and its double *fnb* knockout (P=0.206, Figure 3.5).

To elucidate which of the two FnBPs was responsible for platelet aggregation induced by *S. aureus* strain P1 grown in blood, single *fnbA* and *fnbB* complements of strain DU5998 were used for platelet aggregometry. Complementation of DU5998 with *fnbB* but not *fnbA* reduced lag time, but this was not statistically significant as compared to DU5998 (P=0.368 and P=0.786 respectively, Figure 3.5). Furthermore, DU5998 complementation with plasmid encoding *fnbB* did not restore lag time to levels observed for the wild type strain P1 (Figure 3.5), suggesting that both FnBPA and FnBPB contribute to *S. aureus*-induced platelet aggregation following bacterial growth to stationary phase in blood. The capacity of DU5998 to induce platelet aggregation despite deletion of both *fnb* genes, suggests that other bacterial surface components are involved in *S. aureus*-induced platelet aggregation following growth in blood.

3.3.4 The ability of *S. aureus* to induce platelet aggregation *in vitro* does not predict development of infective endocarditis *in vivo*

Considerable variation has been observed in the ability of clinical *S. aureus* strains to induce platelet aggregation *in vitro*, but it is not known how this correlates with disease association (Rindi *et al.*, 2006). Given that *S. aureus*-induced platelet aggregation is thought to contribute to vegetation formation in infective endocarditis (see Section 1.4.3.3), the association of the capacity of *S. aureus* strains to induce platelet aggregation *in vitro* with the development of infective endocarditis was examined. It was hypothesised that *S. aureus* infective endocarditis strains would have a greater propensity to induce platelet aggregation than either nasal commensal or bacteraemia strains.

For *S. aureus* strains that grew in blood, there was no correlation between the source of *S. aureus* isolation (infective endocarditis, nasal commensal or bacteraemia) and lag time to platelet aggregation at both stationary (P=0.948) and mid-exponential phases of growth (P=0.265, Figure 3.2). Additionally, there was no correlation between *S. aureus* strain origin and lag time to platelet aggregation *in vitro* after growth to stationary (P=0.715) and exponential phase (P=0.235) in BHI (Figure 3.2). Similarly, there was no association between strain origin and maximal percentage platelet aggregation for strains grown to stationary (P=0.253) and mid-exponential phase (P=0.826) in BHI (Figure 3.6).

3.3.5 Thrombus formation during *S. aureus* growth in human blood *ex vivo* does not influence platelet aggregation

Ex vivo thrombus formation was observed during bacterial growth in blood, particularly at the mid-exponential phase of growth. In order to exclude the possibility that presence of a blood clot affected lag time to aggregation (due to plasma protein sequestration within the thrombus), platelet aggregometry was performed using *S. aureus* strain 207 grown to stationary phase in blood, either in the presence or absence of a thrombus in the flask. The presence of a blood clot did not influence lag time to platelet aggregation (P=0.922, Figure 3.7).

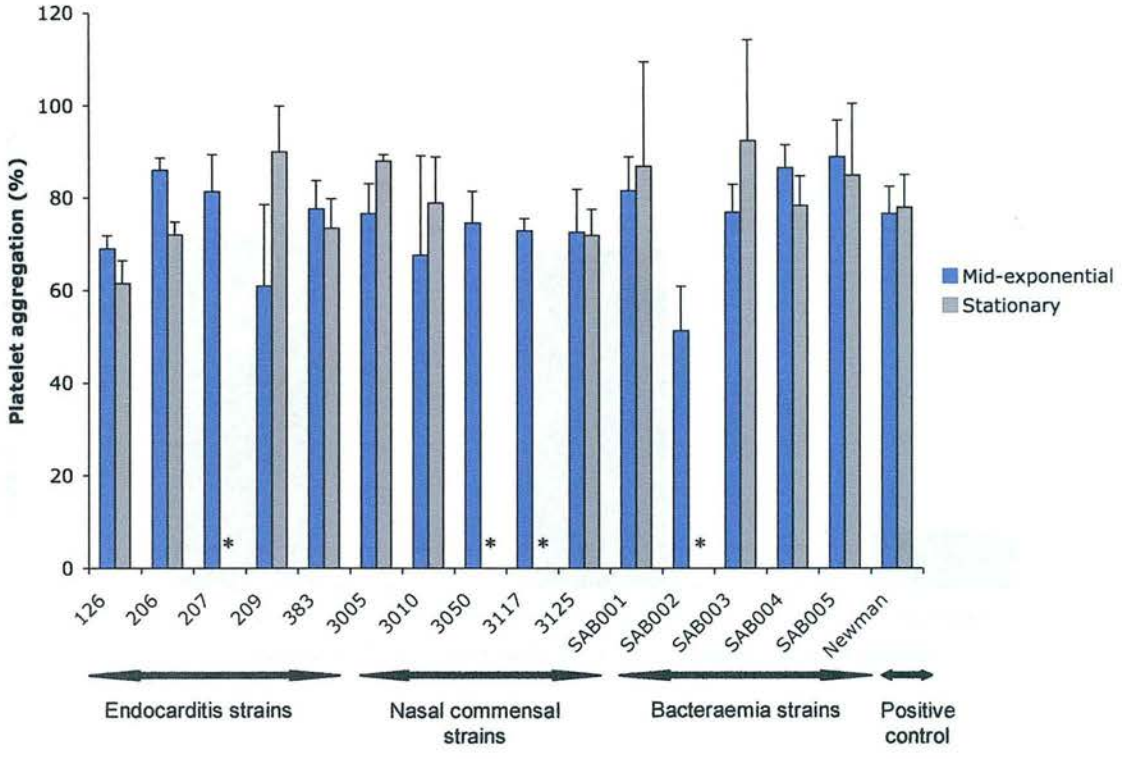

Figure 3.6. Maximal percentage platelet aggregation induced by *S. aureus* clinical strains grown to mid-exponential (blue) and stationary phase (grey) in BHI. Strain Newman served as a positive control. Experiments were performed in triplicate using three different platelet donors.

* Absence of platelet aggregation at 25 min.

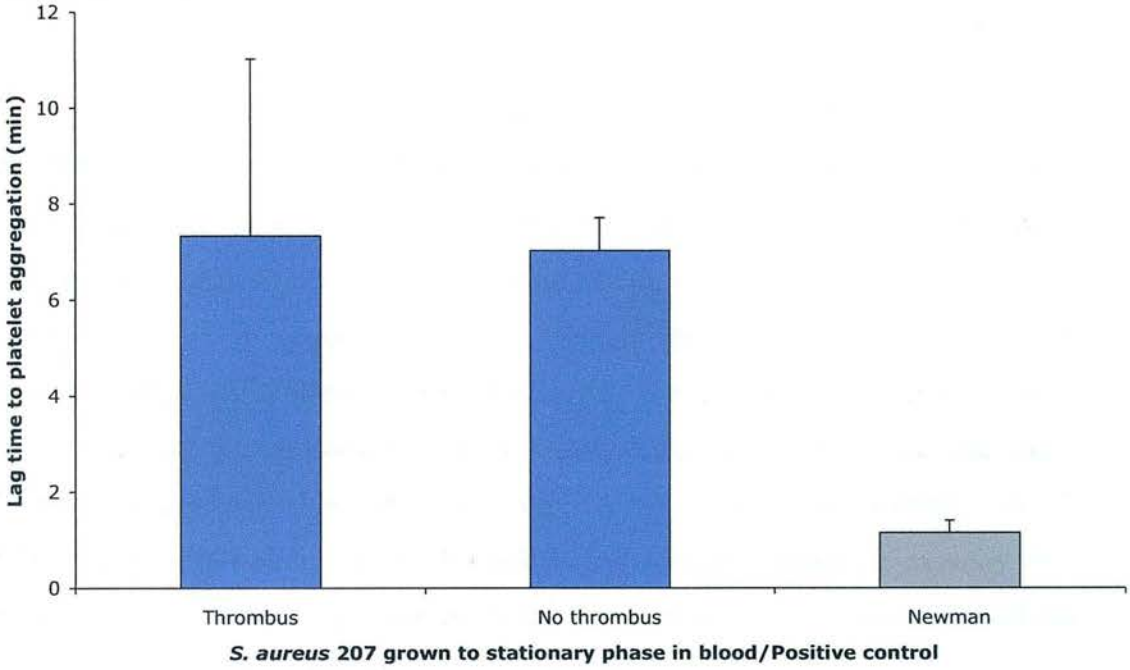

Figure 3.7. Lag time to platelet aggregation induced by *S. aureus* strain 207 grown to stationary phase in whole human blood, either in the presence or absence of a thrombus in the culture. Strain Newman grown to stationary phase in BHI (grey) served as a positive control. Experiments were performed with one blood donor for the growth medium and two different platelet donors for aggregometry.

3.4 Discussion

S. aureus induces platelet aggregation after growth in nutrient broth (Fitzgerald *et al.*, 2006a), but the capacity of *S. aureus* strains grown in human blood to stimulate platelet aggregation has not been previously examined. Bacterial growth in human blood *ex vivo* represents the *in vivo* conditions more closely than nutrient broth, and is likely to have a profound influence on bacterial gene expression and phenotype (Novick, 2003), particularly as recent research has demonstrated variation in *S. aureus* gene transcription and translation following growth in nutrient-deplete media (Clarke *et al.*, 2004, Schlievert *et al.*, 2007, Torres *et al.*, 2006, Wertheim *et al.*, 2008, Wiltshire & Foster, 2001). This is the first study to analyse the capacity of *S. aureus* to induce platelet aggregation following growth in whole human blood and determine its correlation with strain clinical origin.

3.4.1 *S. aureus* growth in whole human blood

Differences were observed in the capacity of *S. aureus* strains to grow in human blood as compared to BHI. Notably, strains grew at a slower rate and to a lower yield in blood, with bacterial counts at the stationary phase of growth similar to those observed by Schlievert *et al*, in their study examining the bacterial factors implicated in toxic shock syndrome (Schlievert *et al.*, 2007). Growth of *S. aureus* is abrogated in other nutrient-deplete media such as Roswell Park Memorial Institute (RPMI) and chemically-defined metal limitation (CL) media and may be due to the low levels of free nutrients, such as iron, in these growth media (Mazmanian *et al.*, 2003, Torres *et al.*, 2006).

There was strain-dependent variation in the ability of *S. aureus* to grow in blood, which has not previously been examined. Specifically, *S. aureus* strains 3010, SAB003, SAB004 and Newman, the latter of which has been widely used as a model strain in functional, genetic and *in vitro* studies (Grundmeier *et al.*, 2004), did not grow in blood. This is consistent with work by Mazmanian *et al*, who reported that

strain Newman failed to grow in media containing less than 300 nM free iron (Mazmanian *et al.*, 2003). It is likely that the ability of strain Newman to grow in blood has been attenuated through long-term serial passage *in vitro*.

It is feasible that genetic variation in factors facilitating iron acquisition influences the ability of clinical *S. aureus* isolates to grow in blood. Haem iron contained within haemoglobin is the preferred iron source for *S. aureus* during growth in minimal media and is obtained via the Isd pathway (see Section 1.3.5.5) (Skaar *et al.*, 2004). *S. aureus* can also scavenge iron within siderophores via staphylococcal siderophore-transporters, staphylococcal iron-regulated uptake and ferric hydroxamate uptake systems (Skaar *et al.*, 2004). It is possible that iron transporters such as the Isd pathway are important for growth in blood, although neither *isdA* nor *isdB* mutations affected the ability of *S. aureus* to grow in blood in the current study. Furthermore, *S. aureus* Newman, which did not grow in blood, expresses functional IsdA, IsdB, IsdC and IsdH (Mazmanian *et al.*, 2003, Mazmanian *et al.*, 2002, Torres *et al.*, 2006). This suggests that variation in genes encoding other factors that facilitate nutrient acquisition or evasion of innate immune defence may modulate *S. aureus* survival in the human bloodstream, and contribute to the strain-dependent variation in growth observed in the current study.

3.4.2 Influence of growth medium on platelet aggregation induced by *S. aureus*

In the current study, all *S. aureus* strains grown in human blood were able to induce platelet aggregation, whereas 4 strains grown in BHI were unable to do so at the stationary phase of growth. This difference in phenotype is most likely due to differential expression of bacterial surface components such as MSCRAMMs following bacterial growth in blood.

The current study indicates that bacterial factors other than ClfA, ClfB, SdrCDE, IsdA, IsdB and SpA mediate platelet aggregation induced by *S. aureus* strains grown

in human blood. Notably, platelet aggregation was attenuated in a strain containing double *fnbA* and *fnbB* gene deletions (DU5998), suggesting that both FnBPs induce platelet aggregation. This is consistent with current knowledge regarding the central role of FnBPs in the pathogenesis of infective endocarditis, as they facilitate *S. aureus* binding to valvular endothelium, while the $A4^{+16}$ domain of FnBPA alone is capable of initiating vegetation formation by a combination of fibrinogen and fibronectin binding (see Section 1.3.5.1) (Heying *et al.*, 2007, Piroth *et al.*, 2008). A putative role of the FnBPs in platelet aggregation during growth in blood may extend the predicted importance of both FnBPA and FnBPB in the pathogenesis of infective endocarditis.

S. aureus DU5998 retained the ability to induce platelet aggregation despite deletion of both *fnb* genes, suggesting that other bacterial factors contribute to *S. aureus*-induced platelet aggregation following growth in blood. Although proteins such as Efb or Pls inhibit fibrinogen binding and platelet aggregation, altered expression of these factors is unlikely to account for the phenotypic effects observed in this work (Juuti *et al.*, 2004, Shannon & Flock, 2004). Specifically, Efb is an anchorless protein which can reattach to the *S. aureus* cell wall after secretion, but is predominantly present in the culture supernatant and is unlikely to be present in high concentrations in the washed bacterial preparations used for aggregometry (Shannon & Flock, 2004). In addition, Pls is encoded on Type I SCC*mec* and is only expressed by MRSA strains, whereas the clinical *S. aureus* isolates used in this study were all MSSA (Juuti *et al.*, 2004, Shannon & Flock, 2004). Furthermore, although *S. aureus* capsule inhibits ClfA-platelet interactions at the stationary phase of growth, it is unlikely to account for the reduced aggregation induced by cultures grown in BHI, as all strains used in these assays retained the ability to bind fibrinogen (Figure 3.3) (Risley *et al.*, 2007). It has also been demonstrated that the presence of capsule does not influence *S. aureus*-induced platelet aggregation (Risley *et al.*, 2007). It is likely that other surface components facilitate *S. aureus*-induced platelet aggregation after growth in blood, but the nature of these factors remains to be identified.

In the current study, there was evidence of altered gene transcription following *S. aureus* growth in blood. Specifically, *clfA* gene transcription was downregulated during growth in blood, consistent with findings by a number of investigators who observed downregulation of *clfA* transcription in animal models of infection (Josefsson *et al.*, 2008, Nanra *et al.*, 2009, Wolz *et al.*, 2002). Meanwhile, the ability of FnBPs to induce platelet aggregation at the stationary phase of growth, suggests that these MSCRAMMs are expressed during stationary phase in blood, although it is not known how transcription and expression levels compare with bacteria grown in nutrient broth (Peacock *et al.*, 1999). Notably, Yarwood *et al* identified increased transcription of *fnbA* following *S. aureus* growth in human serum *ex vivo* in comparison to *S. aureus* grown in nutrient-replete conditions (Yarwood *et al.*, 2001), and transcription of *fnbA* has recently been demonstrated to be independent of *sae* in rabbit models of infective endocarditis (Cheung *et al.*, 2009).

Altered *S. aureus* gene transcription has also been observed in a number of studies using bacteria grown in nutrient–deplete media (Pishchany *et al.*, 2009, Torres *et al.*, 2006, Wertheim *et al.*, 2008). Transcription of genes encoding Isd proteins is upregulated after growth in minimal media (Pishchany *et al.*, 2009, Torres *et al.*, 2006), while ClfB is present on the *S. aureus* cell surface late into stationary phase when strains are grown in minimal, but not nutrient-rich media (Wertheim *et al.*, 2008). However, deficiencies in IsdA, IsdB and ClfB did not alter platelet aggregation in the current work. Other studies have examined alteration in global *S. aureus* gene transcription during growth in serum *in vitro*, and have identified upregulation of genes involved in amino acid and antimicrobial peptide synthesis, with downregulation of *agr* (Wiltshire & Foster, 2001, Yarwood *et al.*, 2001).

Schlievert *et al* examined changes in transcription and translation following *S. aureus* growth in heparinised whole human blood (Schlievert *et al.*, 2007). Growth of a range of *S. aureus* strains in human blood was associated with reduced production of TSST-1, α- and δ-toxins, lipase and staphylococcal enterotoxin C (Schlievert *et al.*, 2007). Alterations in gene transcription were mediated by the effects of the α- and β-globin chains of haemoglobin on transcriptional regulators

SrrA-SrrB and *agr* (see Section 1.3.6) (Schlievert *et al.*, 2007). *S. aureus* interactions with host tissue also influence bacterial gene expression, as in the presence of endothelial cells, *S. aureus* genes involved in amino acid and peptide transport, peptidase synthesis, biosynthesis and DNA repair are upregulated (Vriesema *et al.*, 2000).

A number of investigators have identified altered transcription of selected *S. aureus* genes following bacterial growth *in vivo*. Goerke *et al* used qRT-PCR to analyse the transcription of *agr* in *S. aureus* isolates obtained from the sputum of patients with cystic fibrosis (Goerke *et al.*, 2000). They identified low levels of RNAIII expression within sputum, which unlike growth in nutrient broth, did not influence transcription of genes encoding α-toxin and SpA (Goerke *et al.*, 2000). The genes encoding IsdA, IsdB, SdrD and SpA are expressed *in vivo* in animal models of infection, but the role of these MSCRAMMs in the pathogenesis of infective endocarditis in humans is unknown (Cheng *et al.*, 2009, Pishchany *et al.*, 2009, Torres *et al.*, 2006). In addition, σ^B, the transcriptional regulator of ClfA, plays a lesser role *in vivo* than the regulator *sae*, which upregulates expression of ECM-binding proteins Eap, Emp, FnBPA and α-toxin (Cheung *et al.*, 2009, Goerke *et al.*, 2005, Harraghy *et al.*, 2005, Xiong *et al.*, 2006). It has already been demonstrated that FnBPA and α-toxin induce platelet aggregation, but it is not known whether Eap or Emp are able to do so (Bhakdi *et al.*, 1988, Piroth *et al.*, 2008). However, Emp has been implicated in biofilm formation under low iron conditions and may potentially contribute to vegetation formation (Johnson *et al.*, 2008).

DNA microarrays can be used to elucidate genome-wide variation in *S. aureus* gene transcription following growth in human blood. For example, Group A and B streptococcal growth in blood was associated with increased expression of factors facilitating survival in the host, including those enabling immune evasion, carbohydrate and amino acid metabolism, adhesins and transcriptional and stress-response regulators (Graham *et al.*, 2005, Mereghetti *et al.*, 2008). Transcriptomic analysis of *S. aureus* grown in human blood has not been performed to date, but results from the current study demonstrate that this is imperative to elucidate the

bacterial factors essential for pathogen survival in the human host. Furthermore, if genes encoding surface factors are upregulated following bacterial growth in blood, these may account for the observed variation in the ability of *S. aureus* strains to induce platelet aggregation after growth in different media *in vitro*, and may ultimately predict the factors implicated in infective endocarditis pathogenesis *in vivo*.

3.4.3 Influence of strain clinical origin on platelet aggregation induced by *S. aureus*

Correlations have been identified between the capacity of *S. aureus* and *S. sanguinis* to stimulate platelet aggregation *in vitro* and induce infective endocarditis in animal models (Herzberg *et al.*, 1992, Kessler *et al.*, 1987, Sullam *et al.*, 1996). For example, Kessler *et al* performed platelet aggregometry using a range of organisms and found the lag time to be significantly reduced in strains causing infective endocarditis compared to bacteraemia (Kessler *et al.*, 1987). However, it is unclear whether such an association is observed with strains isolated from human infection.

The relationship between strain disease association in humans and lag time or percentage aggregation was examined in the current study. There were no differences in platelet aggregation induced by *S. aureus* infective endocarditis, nasal commensal and bacteraemia strains, regardless of growth phase or growth medium. These observations are consistent with our understanding of the population genetics of *S. aureus*, which indicate that there are no significant differences in pathogenic potential between commensal and clinical isolates. For example, Feil *et al* used MLST to demonstrate that *S. aureus* genotype did not correlate with ability to cause disease *in vivo* in a study examining 334 commensal and infection strains (Feil *et al.*, 2003). Similarly, Lindsay *et al* did not identify any variation in gene prevalence between 100 commensal and 61 invasive isolates using microarrays (Lindsay *et al.*, 2006). However, other investigators have reported that strains of clonal lineage CC30 were more prevalent in invasive disease than colonisation (Wertheim *et al.*,

2005b). It is commonly thought that most *S. aureus* invasive diseases occur following infection by nasal commensal strains (van Belkum *et al.*, 2009), which may account for the lack of association between strain genotype, platelet aggregation and disease causation both in the current study and in previously published research (Feil *et al.*, 2003, Lindsay *et al.*, 2006).

Results from the current research also suggest that the ability of isolates to induce platelet aggregation *in vitro* does not predict development of infective endocarditis in patients with bacteraemia. These observations are consistent with findings by Fowler *et al* in their study analysing the contribution of host and pathogen factors to adverse outcome in 324 cases of *S. aureus* bacteraemia, where there was no correlation between MSCRAMM gene prevalence and development of haematogenous complications of *S. aureus* bacteraemia (Fowler et al., 2005a). Conversely, the same investigators found that patients with haematogenous complications of *S. aureus* bacteraemia were more likely to be infected with *S. aureus* strains of clonal lineages CC5 and CC30, than bacteraemic patients without disseminated infection (Fowler *et al.*, 2007). However, it is not known whether strain clonal origin correlated with expression levels of bacterial surface components in either of these studies (Fowler *et al.*, 2005a, Fowler *et al.*, 2007).

Collectively, these results suggest that the ability of *S. aureus* to induce platelet aggregation *in vitro* does not correlate with development of infective endocarditis *in vivo*, possibly due to MSCRAMM redundancy (see Section 1.3.5), indicating that other host and pathogen factors may influence susceptibility to infective endocarditis. For example, the ability of *S. aureus* strains to induce infective endocarditis may depend on their ability to secrete toxins or evade immune defences, for example, by prevention of platelet microbicidal protein (PMP) release (see Section 1.5.2). In support of this theory, Ruotsalainen *et al* discovered that *S. aureus* strains associated with infective endocarditis had reduced haemolytic activity, which may result in reduced PMP release, enhanced bacterial survival and bacterium-platelet interactions, facilitating vegetation development *in vivo* (Ruotsalainen *et al.*, 2008). In keeping with this, the ability of bacteria to resist PMP correlates with the

development of staphylococcal and streptococcal infective endocarditis in humans (Wu *et al.*, 1994).

Host factors such as variation in innate immunity, platelet activation and platelet receptor polymorphisms may have a more profound influence on the outcome of bacterium-platelet interactions than pathogen factors (Brouwer *et al.*, 2009, Gawaz *et al.*, 1995, Meisel *et al.*, 2004). Alternatively, the lack of a correlation between strain clinical origin and *in vitro* platelet aggregation in the current study may reflect the low shear conditions in the platelet aggregometer, which differ from the high shear environment found near the valve surface *in vivo* (Kerrigan *et al.*, 2007). It is also feasible that some of the infective endocarditis isolates used in the current study were not associated with vegetations, as is the case with 5% to 25% of cases of infective endocarditis (De Castro *et al.*, 1997, Di Salvo *et al.*, 2001), accounting for the lack of a correlation with *in vivo* platelet aggregation. It is evident from these results that the clinical outcome of infection depends upon the dynamic interaction between bacterial and host factors, rather than each in isolation.

3.4.4 Host-pathogen interactions contributing to platelet aggregation and thrombus formation

Thrombus formation was observed within the flasks used for bacterial culturing. The factors contributing to this blood clot are unknown, but may include MSCRAMMs facilitating bacterium-platelet binding, siaylated protein inducing bacterium-erythrocyte interactions, the effects of coagulase, or downregulation of the fibrinolytic effects of staphylokinase and aureolysin (Beaufort *et al.*, 2008, Bokarewa *et al.*, 2006, Fitzgerald *et al.*, 2006a, Shin *et al.*, 2005). Notably, thrombi were less evident during the stationary phase of growth, possibly due to the actions of staphylokinase, which is expressed during the late-exponential phase of growth and dissolves blood clots (Bokarewa et al., 2006).

It would be intriguing to further characterise this possible '*ex vivo* vegetation' in real-time, to further elucidate the host and pathogen factors contributing to its development. Further understanding of bacterium-induced thrombus formation *ex vivo* may help identify relevant factors mediating vegetation formation *in vivo*. Although some insight into bacterium-platelet interactions has been gained from *in vitro* assays, it is known that bacteria in free suspension behave differently from those present in a biofilm which may exist in a vegetation (George *et al.*, 2009). For example, *S. aureus* strains grown to mid-exponential phase in biofilms *in vitro* exhibit a greater ability to bind immobilised platelets at low shear rates than suspension-grown cells, while the converse is true at the stationary phase of growth under high shear conditions (George *et al.*, 2009). The *ex vivo* thrombus identified in the current study may represent a novel model for the analysis of host-pathogen interactions in infective endocarditis, particularly as qRT-PCR has recently been used to determine inter-vegetation bacterial gene transcription in a rabbit model of infective endocarditis (Cheung *et al.*, 2009).

3.4.5 Conclusions

In summary, this study demonstrates that *S. aureus* growth in blood significantly alters bacterial gene expression and phenotype. All *S. aureus* strains grown in blood induced platelet aggregation and this process may involve FnBPA, FnBPB and other bacterial surface components at the stationary phase of growth. The current data are more likely to be representative of the *in vivo* situation than previous studies that used nutrient broth as a growth medium. However, this research was limited by the low shear conditions present in the aggregometer. Future studies should incorporate the use of high shear systems such as parallel-plate flow chambers and cone-and-plate rheometers to characterise the dynamic host-pathogen interactions contributing to vegetation formation in infective endocarditis (Mascari & Ross, 2003, Pawar *et al.*, 2004). Such studies should also attempt to simulate *in vivo* conditions as far as possible, which include culturing bacteria in the presence of *ex vivo* materials such as human blood.

The nature of the bacterial factors implicated in platelet aggregation induced by *S. aureus* strains grown in blood has not been fully characterised, but may be identified by further experiments with deletion mutant strains, in parallel to the use of expression microarray analysis. For example, creation of strains deficient in the expression of FnBPA, FnBPB and other bacterial surface components, in the background of the 4 *S. aureus* strains that were able to induce platelet aggregation after growth in blood but not BHI, would indicate their role in the stimulation of platelet aggregation by those strains following growth in blood.

It is clear that global transcriptomic and proteomic studies of organisms that are common causes of infective endocarditis, such as staphylococci, streptococci and enterococci (Moreillon & Que, 2004) grown in human blood, within a thrombus or in the presence of host tissue such as valvular endothelium, are now necessary to determine the factors implicated in bacteraemia and infective endocarditis. Such studies may result in the identification of novel targets for vaccine or antimicrobial development in the fight against invasive bacterial infection.

CHAPTER 4

INVESTIGATION INTO THE ASSOCIATION OF PLATELET ACTIVATION WITH CLINICAL OUTCOME IN INFECTIVE ENDOCARDITIS

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4.1 Introduction

Platelet activation is increased in prothrombotic conditions such as unstable angina and myocardial infarction (MI) (Sarma *et al.*, 2002). Considering the importance of vegetations in the pathogenesis of infective endocarditis, and the contribution of bacterium-induced platelet activation and aggregation to this process (Di Salvo et al., 2001, Fitzgerald et al., 2006a, Herzberg et al., 1992, Sullam et al., 1996), it was hypothesised that *in vivo* platelet activation would be raised in patients with infective endocarditis. Platelet activation was determined using well-characterised flow cytometric markers, and levels compared to those obtained from age- and sex-matched healthy volunteers and patients with bacteraemia, to take into account the effects of age, gender, sepsis and antibiotic treatment on platelet function (Faraday *et al.*, 1997, Gawaz *et al.*, 1995, Russwurm *et al.*, 2002, Shattil *et al.*, 1980).

4.2 Methods

Forty-four patients with infective endocarditis (73% male, 56±17 years), 4 patients with *S. aureus* bacteraemia (75% male, 46±28 years) and 30 age- and sex-matched healthy volunteers (70% male, 54±17 years) were recruited into the study between November 2006 and April 2009 using the inclusion and exclusion criteria outlined in Section 2.1. Clinical data were collected prospectively for patients with infective endocarditis and outcomes defined as described in Section 2.2. Transthoracic and/or transoesophageal echocardiography were performed on patients with infective endocarditis and bacteraemia, and analysed as outlined in Section 2.3.

Venepuncture was performed on all recruited subjects as described in Section 2.4, with 3 ml of blood drawn into a tube containing the anticoagulant PPACK for determination of whole blood platelet-monocyte aggregates (PMA) and platelet P-selectin expression as outlined in Section 2.10.2.

4.3 Results

4.3.1 Characteristics of the study population

Baseline characteristics of recruited patients with infective endocarditis are outlined in Table 4.1. The majority of patients had community-acquired infection of native valves and at least one risk factor for the development of infective endocarditis. Notably, 20% had underlying congenital heart disease, 16% had a previous episode of infective endocarditis and 11% were intravenous drug abusers. Of the patients with infective endocarditis, 36% were immunocompromised due to underlying disease, drug therapy or alcohol abuse. Additionally, 30% of patients had an identified source of infection, with most cases associated with arterial or venous line placement or recent invasive dental work. A past medical history of arterial or venous thromboembolism unrelated to infective endocarditis, defined as a documented history of MI, unstable angina, ischaemic cerebrovascular disease, peripheral vascular disease, deep venous thrombosis or pulmonary embolism, was evident in 48% of patients.

Of the infective endocarditis study population, 23 subjects (52%) were transferred to the Edinburgh Royal Infirmary (ERI) from district general hospitals for consideration of surgery. There was an approximate 3- to 4-week delay between symptom onset and patient presentation, but clinical diagnosis and institution of antibiotic therapy occurred within 2 d of presentation (Table 4.1). One subject was recruited prior to antibiotic therapy, the remainder being recruited approximately 2 weeks after the introduction of antimicrobial therapy. Peripheral blood samples were obtained with a 19-gauge needle in most patients.

Table 4.1. Baseline characteristics of 44 patients with infective endocarditis at the time of recruitment

Demographic and risk factors of the infective endocarditis study population	Number (%)
Male	32 (73%)
Age, years	56±17
Community acquired	35 (80%)
Risk factors for infective endocarditis	
Prosthetic valve	15 (34%)
Bioprosthetic	8
Mechanical	7
Early prosthetic valve infective endocarditis	2 (5%)
Valvular heart disease *	16 (36%)
Congenital heart disease	9
Bicuspid aortic valve	4
Rheumatic heart disease	2
Degenerative valve	2
Previous episode of infective endocarditis	7 (16%)
Intravenous drug abuser	5 (11%)
Immunocompromised *	16 (36%)
Alcoholism	9
Disease	7
Immunosuppressive drug therapy	4
Identified source of infection	13 (30%)
Skin	7
Dental	3
Bowel	2
Prostate	1

Demographic and risk factors of the infective endocarditis study population	Number (%)
Recent vascular instrumentation	6 (14%)
Past medical history	
Dialysis dependent end stage renal failure	2 (5%)
Past history of thromboses	21 (48%)
Arterial	16
Venous	3
Arterial + venous	2
Time from symptom onset to presentation, d	23 ± 38
Time from symptom onset to diagnosis, d	25 ± 40
Recruitment	
Blood sampling via peripheral venepuncture	35 (80%)
Time from diagnosis to recruitment, d	12±14
Days of antibiotics prior to recruitment, d	14±14

* Sub-categories are not mutually exclusive and patients may have had more than one cause of valvular heart disease or reason for being immunocompromised.

Table 4.2 contains patient data from routine haematology and biochemistry blood tests at the time of recruitment and electrocardiograph (ECG) results during the course of their in-patient stay. Most patients were anaemic with some degree of intravascular fluid depletion as evidenced by raised haematocrit levels. There was marked variation in platelet count, CRP, urea and creatinine levels. The mean white cell count, electrolyte levels, body temperature, mean arterial pressure and heart rate were within normal limits. First, second or third degree atrioventricular heart block was present in 41% of patients, while 27% of subjects developed atrial fibrillation during the course of their illness.

Results of blood and histopathological cultures are outlined in Table 4.3. Valvular tissue from 18 patients was sent for histopathology, of which 8 had positive results. Unlike several recent studies, *Streptococcus* was the most frequently isolated organism, and included viridans streptococci (*S. mitis, S. oralis, S. sanguinis, S. gordonii* and *Streptococcus salivarius*), as well as species *Streptococcus bovis* and *Streptococcus agalactiae*. Of the 4 identified *S. bovis* infective endocarditis cases in the current study, 2 were associated with benign colonic polyps, while none were associated with colorectal cancer (Bashore *et al.*, 2006). Staphylococcal species that were isolated include *S. aureus, Staphylococcus hominis, Staphylococcus capitis, Staphylococcus epidermidis* and *Staphylococcus lugdunensis*. *Enterococcus spp.* were isolated from 4 patients, while *Aerococcus urinae, Aerococcus viridans, Enterobacter cloacae, Gemella sanguinis*, *Granulicatella adiacens, Haemophilus parainfluenzae, Propionibacterium acnes, S. marcescens* and *Stenotrophomonas maltophilia* were isolated from blood or valve cultures from a single patient each. Four patients had polymicrobial infection, predominantly involving culture positivity for different species of staphylococci and streptococci. Blood cultures were negative in 7% of subjects.

Table 4.2. Results of blood tests (haematology and biochemistry) and electrocardiographs (ECGs) in 44 recruited patients with infective endocarditis.

Investigation results for the infective endocarditis study population	Number (%)	Reference range *
Baseline blood results at time of admission to or diagnosis at ERI		
Haemoglobin, g/l	99 ± 14	130 – 180 (M) 115 – 165 (F)
Haematocrit, %	30.5 ± 4.0	40 – 54 (M) 37 – 47 (F)
White cell count, x 10^9/l	10.0 ± 5.0	4.0 – 11.0
Platelet count, x 10^9/l	311 ± 127	150 – 350
CRP, mg/l	80 ± 68	< 5
Venous HCO_3, mmol/l	25 ± 8	22 – 30
Sodium, mmol/l	136.8 ± 3.0	135 – 145
Potassium, mmol/l	4.3 ± 0.6	3.6 – 5.0
Creatinine, μmol/l	117 ± 129	60 – 120
Urea, mmol/l	5.5 ± 3.5	2.5 – 6.6
Temperature, °C	36.9 ± 0.4	N/A
Mean arterial pressure, mmHg	84 ± 9	N/A
Heart rate, beats per minute	88 ± 12	N/A
Positive ECG findings		N/A
Atrioventricular block	18 (41%)	
Atrial fibrillation	11 (27%)	
Paced rhythm	2 (5%)	

Values represent the mean ± standard deviation. The mean temperature, arterial pressure and heart rate were determined once weekly during the patient's admission up to a maximum 6-week period. * Reference ranges for blood tests performed at the Edinburgh Royal Infirmary (ERI) clinical laboratories were taken from the Lothian University Hospitals Division Department of Laboratory Medicine Guidelines for Users of Biochemistry and Haematology (ERI), 2009 edition, v 4.2. M, male; F, female; CRP, C-reactive protein. N/A, not applicable.

Table 4.3. Microbiological and immunological results in 44 patients with infective endocarditis.

Microbiological and immunological findings	Number of subjects (%)
Histopathological criteria (n=18) *	8 (44%)
Organisms †	
Streptococci	17 (39%)
S. mitis	5
S. bovis	4
S. oralis	3
S. sanguinis	2
S. salivarius	1
S. gordonii	1
S. agalactiae	1
Staphylococci	16 (36%)
S. aureus	10
S. epidermidis	3
S. hominis	1
S. capitis	1
S. lugdunensis	1
Enterococcus faecalis/faecium	4 (9%)
Others #	9 (20%)
Polymicrobial ‡	4 (9%)
Culture negative	3 (7%)
Immunological phenomena ∫	18 (41%)

* Histopathological data was available for 18 patients. † Sub-groups for causative organism are not mutually exclusive. # Other isolated organisms include *Aerococcus urinae, Aerococcus viridans, Enterobacter cloacae, Gemella sanguinis, Granulicatella adiacens, Haemophilus parainfluenzae, Propionibacterium acnes, Serratia marcescens, Stenotrophomonas maltophilia,* (n=1 for each). ‡ Polymicrobial infections were caused by *S. capitis* + *S. epidermidis, S. oralis* + *S. hominis, S. lugdunensis* + *S. mitis, S. aureus* + *E. faecalis.* ∫ Immunological phenomena identified include rashes, splinter haemorrhages, arthropathy and Janeway's lesions.

Transthoracic or transoesophageal echocardiograms for diagnosis of infective endocarditis were performed at an average of 3±3 d after patient presentation and results outlined in Table 4.4. Sixteen patients had transthoracic echocardiograms alone, 5 had transoesophageal echocardiograms and 18 subjects had both. The mitral valve was most commonly affected (41%), followed by the aortic valve, either alone (30%) or in combination with the mitral valve (16%), and the tricuspid or pulmonary valves were involved in 13% of cases of infective endocarditis. Vegetations were identified in 77% of patients, fistulae in 14% and abscesses in one quarter of cases. Approximately one-third of patients with vegetations had multiple vegetations. Valve destruction due to dehiscence or perforation occurred in 17 subjects, while new valvular regurgitation occurred in 86% of cases.

Transthoracic and transoesophageal echocardiograms used for measurement of vegetation length, width and mobility in the current study were performed at a mean of 7±8 d after diagnosis and were available for 32 of the 34 patients with vegetations (94%). Nine subjects had transthoracic echocardiograms alone, 6 had transoesophageal echocardiograms and 14 patients had both imaging modalities performed. Mean vegetation length and width were 15 ± 7 mm and 9 ± 4 mm respectively, and over 80% of vegetations were mobile.

Table 4.4. Echocardiogram results in 44 patients with infective endocarditis.

Echocardiogram results	Number of subjects (%)
Valve(s) affected	
Mitral	18 (41%)
Aortic	13 (30%)
Aortic + mitral	7 (16%)
Tricuspid ± aortic/mitral valve	5 (11%)
Pulmonary	1 (2%)
Vegetation(s)	34 (77%)
Multiple vegetations	10
Vegetation length (n=32) *	15 ± 7 mm
Vegetation width (n=32) *	9 ± 4 mm
Vegetation mobility (n=32) *	
None	6 (19%)
Low	12 (38%)
Moderate	5 (15%)
Severe	9 (28%)
Fistula	6 (14%)
Abscess	11 (25%)
New valvular regurgitation	38 (86%)
Mild	7
Moderate	9
Severe	22
Valve destruction	17 (39%)

* Characteristics of the largest vegetation were determined on transoesophageal and/or transthoracic echocardiograms in 32 of the 34 patients with infective endocarditis.

Treatment modalities in the infective endocarditis cohort are outlined in Table 4.5. All patients were treated with appropriate antibiotics according to culture sensitivities, mainly comprising of benzylpenicillin in combination with gentamicin. Surgical treatment in the form of valve replacement or repair was performed in 59% of subjects, main indications being the presence of heart failure, emboli, abscesses or fistulae and poor response to medical therapy. A large number of patients were treated with anti-platelets or anticoagulants, but there was no consistency in their use in the management of infective endocarditis.

As outlined in Table 4.6, 41% of patients developed acute renal failure during the course of their infection, while approximately one-third of patients developed heart failure or multi-organ failure alone. Septic emboli that were identified on non-invasive radiological examinations performed at the clinician's discretion, complicated infective endocarditis in 41% of patients, with most cases affecting the cerebral vasculature. Relapses or recurrences occurred in 11% of cases during the 3-month follow-up period, while the mortality rate attributed to infective endocarditis over the same time period was 23%.

Table 4.5. Treatment modalities in the 44 patients with infective endocarditis.

Treatment	Number of subjects (%)
Antibiotics * †	
Penicillins	31 (70%)
Aminoglycosides	26 (59%)
Vancomycin	11 (25%)
Rifampicin	10 (23%)
Cephalosporins	2 (5%)
Others #	4 (9%)
Surgery	26 (59%)
Replacement	20
Repair	5
Replacement + repair	1
Anti-platelets *	17 (39%)
Aspirin	15
Clopidogrel	1
NSAID	1
Anticoagulants * †	26 (59%)
Low-molecular-weight heparin	11
Unfractionated heparin	9
Warfarin	9

* Antibiotic, anticoagulant or anti-platelet therapy was documented for the time of recruitment. † Treatment with different sub-groups of antibiotics and anticoagulants was not mutually exclusive. # Other antimicrobials used in the treatment of infective endocarditis included meropenem in 2 patients, ciprofloxacin in 1 patient and daptomycin in 1 patient. NSAID, non-steroidal anti-inflammatory drug

Table 4.6. Complications in 44 recruited patients with infective endocarditis.

Complications of infective endocarditis	Number of subjects (%)
Heart failure	13 (30%)
Acute renal failure	18 (41%)
Multi-organ failure	13 (30%)
Embolism *	18 (41%)
Cerebral	10
Splenic	4
Pulmonary	3
Renal	2
Spinal	1
Relapse or recurrence	5 (11%)
Death	14 (23%)

* The presence of embolic complications was determined from patients' clinical notes. Two patients had more than one site of septic embolism.

4.3.2 Platelet activation is reduced in patients with infective endocarditis

Platelet activation was determined by measurement of percentage whole blood PMA and platelet P-selectin expression in 44 (100%) and 41 (93%) of the infective endocarditis cohort, respectively. Patients with infective endocarditis had PMA levels of 26.8 ± 16.0 % and platelet P-selectin expression levels of 3.7 ± 3.3 %. PMA did not vary between patients with infective endocarditis, bacteraemia or age- and sex-matched healthy volunteers (P=0.576, Figure 4.1A), and there was no difference in platelet activation between patients with infective endocarditis or bacteraemia caused by *S. aureus* alone (P=0.385 for PMA and P=0.877 for platelet P-selectin expression, data not shown). However, platelet P-selectin expression was reduced in patients with infective endocarditis in comparison to healthy volunteers (P=0.003, Figure 4.1B).

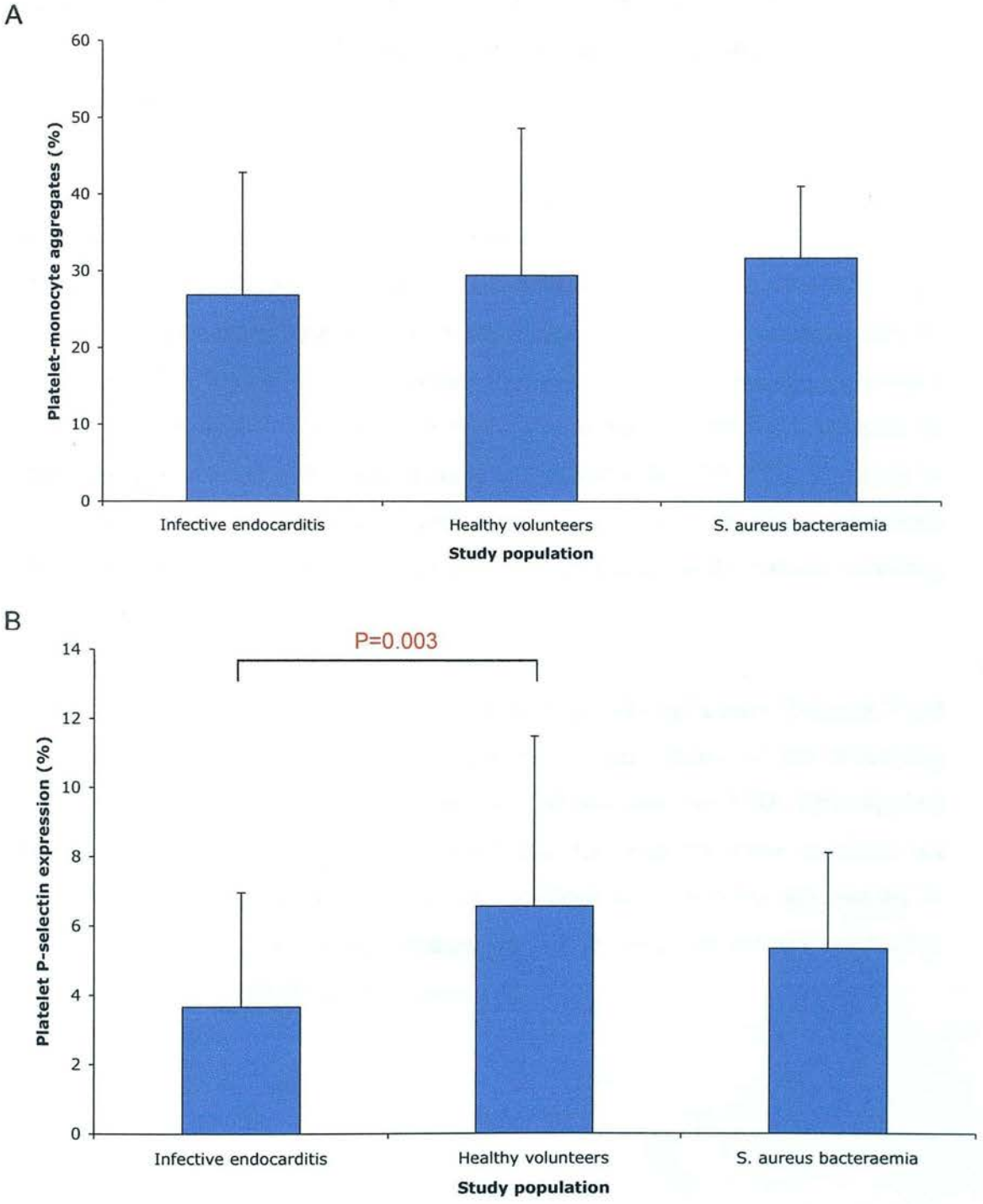

Figure 4.1. Comparison of percentage platelet-monocyte aggregate formation (A) and platelet P-selectin expression (B) between patients with infective endocarditis (n=44), healthy volunteers (n=30) and patients with *S. aureus* bacteraemia (n=4).

4.3.2.1 Platelet activation is increased in intravenous drug abusers and patients with staphylococcal and culture-negative infective endocarditis

To determine possible reasons for reduced platelet activation in the infective endocarditis cohort, the association between platelet activation and clinical characteristics was examined (Tables 4.7 and 4.8). Intravenous drug abuse and a past history of venous thromboses were both associated with increased platelet P-selectin expression ($P<0.001$ and $P=0.012$ respectively, Table 4.8). Additionally, there was a trend towards increased platelet activation in patients with positive blood cultures for staphylococci or those with negative blood cultures ($P=0.053$ for PMA, Figure 4.2A and $P=0.057$ for platelet P-selectin expression, Figure 4.2B). Antibiotic treatment also influenced platelet P-selectin expression, which was lower for patients receiving aminoglycosides ($P=0.009$, Figure 4.3).

Importantly, anti-platelet therapy did not influence platelet activation (Tables 4.7 and 4.8). There were no correlations between gender, age, nature of the underlying valve, presence of valvular heart disease, route of venepuncture, CRP, anticoagulant therapy and platelet activation (Tables 4.7 and 4.8, data for linear variables not shown). Duration of antibiotic treatment correlated with PMA but not platelet P-selectin expression, however, this relationship was not linear and instead appeared as a scattergram ($P=0.049$, $R^2=0.089$, Figure 4.4).

Table 4.7. Correlation between percentage platelet-monocyte aggregates (PMA) and clinical characteristics in 44 patients with infective endocarditis.

Clinical variable	Yes	No	P value
Male gender	26.9 ± 15.4	26.9 ± 19.1	0.966
Prosthetic valve	29.6 ± 20.3	25.6 ± 14.0	0.659
Bioprosthetic	28.5 ± 16.3	30.7 ± 25.0	0.906
Valvular heart disease	26.0 ± 19.8	27.5 ± 14.3	0.479
Intravenous drug abuse	33.8 ± 9.9	26.2 ± 16.8	0.232
Past history of thromboses	28.3 ± 12.3	25.6 ± 19.6	0.472
Arterial	25.2 ± 10.2	27.2 ± 18.8	0.772
Peripheral venepuncture	28.8 ± 17.2	20.3 ± 11.2	0.113
Anti-platelets	24.9 ± 14.4	28.2 ± 17.6	0.449
Anticoagulants	28.0 ± 17.1	25.3 ± 15.4	0.719

Yes indicates that the clinical characteristic was present in the patient sub-group; no indicates that the clinical characteristic was absent.

Table 4.8. Correlation between percentage platelet P-selectin expression and clinical characteristics in 44 patients with infective endocarditis.

Clinical variable	Yes	No	P value
Male gender	4.2 ± 3.4	3.3 ± 3.0	0.435
Prosthetic valve	3.8 ± 3.2	4.0 ± 3.4	0.762
Bioprosthetic	3.1 ± 2.8	4.4 ± 3.6	0.752
Valvular heart disease	3.5 ± 2.8	4.2 ± 3.5	0.524
Intravenous drug abuse	9.9 ± 3.3	3.3 ± 2.5	< 0.001
Past history of thromboses	4.3 ± 3.9	3.6 ± 2.6	0.950
Arterial	2.9 ± 2.9	8.5 ± 2.8	0.012
Peripheral venepuncture	4.1 ± 3.3	3.4 ± 3.3	0.478
Anti-platelets	3.1 ± 2.4	4.5 ± 3.7	0.227
Anticoagulants	3.7 ± 2.8	4.3 ± 3.9	0.826

Yes indicates that the clinical characteristic was present in the patient sub-group; no indicates that the clinical characteristic was absent.

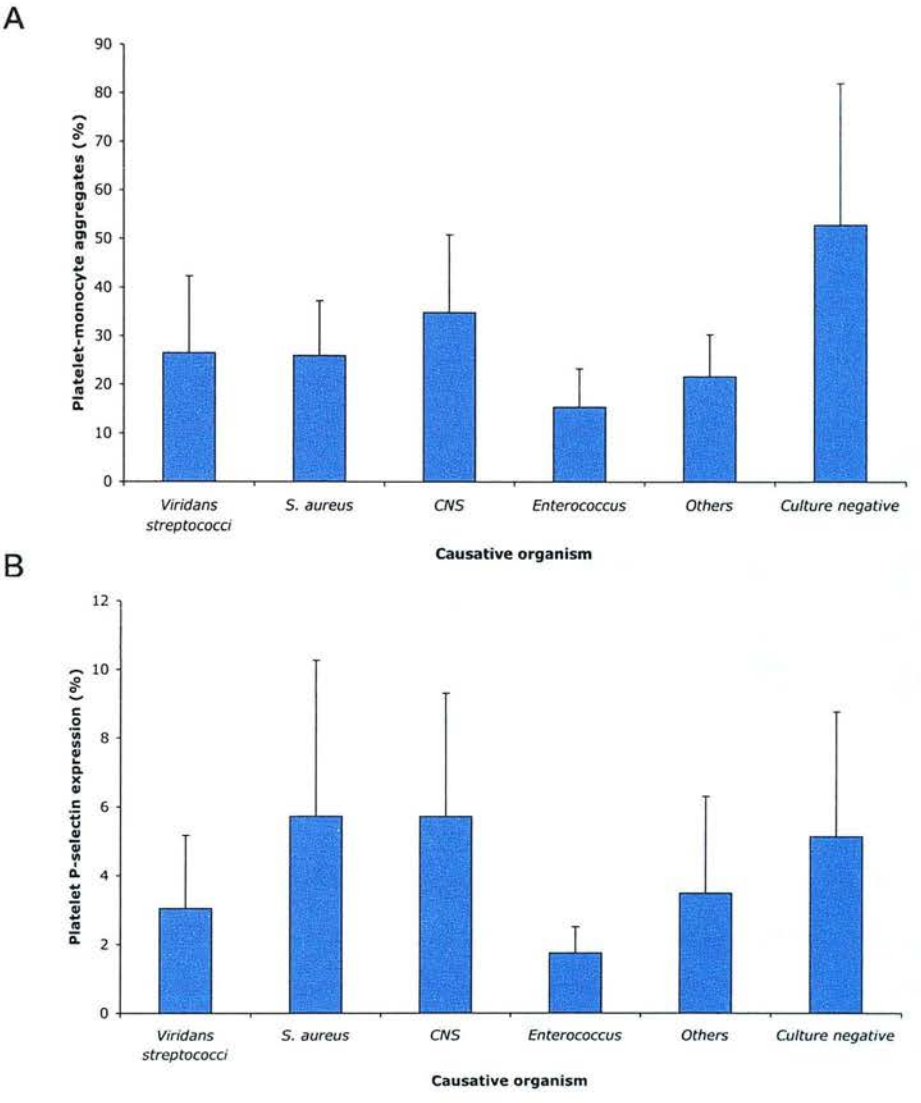

Figure 4.2. Effects of causative organism, treated as a group, on percentage platelet-monocyte aggregates, P=0.053 (A) and percentage platelet P-selectin expression, P=0.057 (B) in 44 patients with infective endocarditis. Post-hoc analysis could not be performed due to the limited study population size. CNS, coagulase-negative staphylococci; other organisms, *Aerococcus urinae, Aerococcus viridans, Enterobacter cloacae, Gemella sanguinis, Granulicatella adiacens, Haemophilus parainfluenzae, Propionibacterium acnes, Serratia marcescens, Stenotrophomonas maltophilia*.

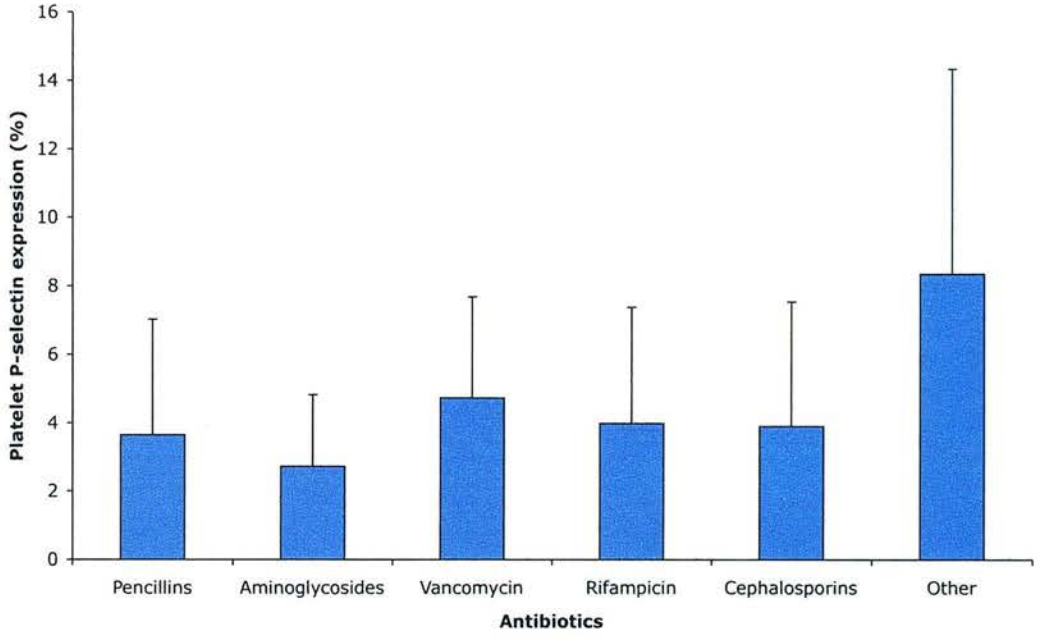

Figure 4.3. Effect of antibiotic treatment on percentage platelet P-selectin expression in patients with infective endocarditis, P=0.009 when treated as a group. Post-hoc analysis could not be performed due to the limited study population size. Other antibiotics include meropenem, ciprofloxacin, daptomycin.

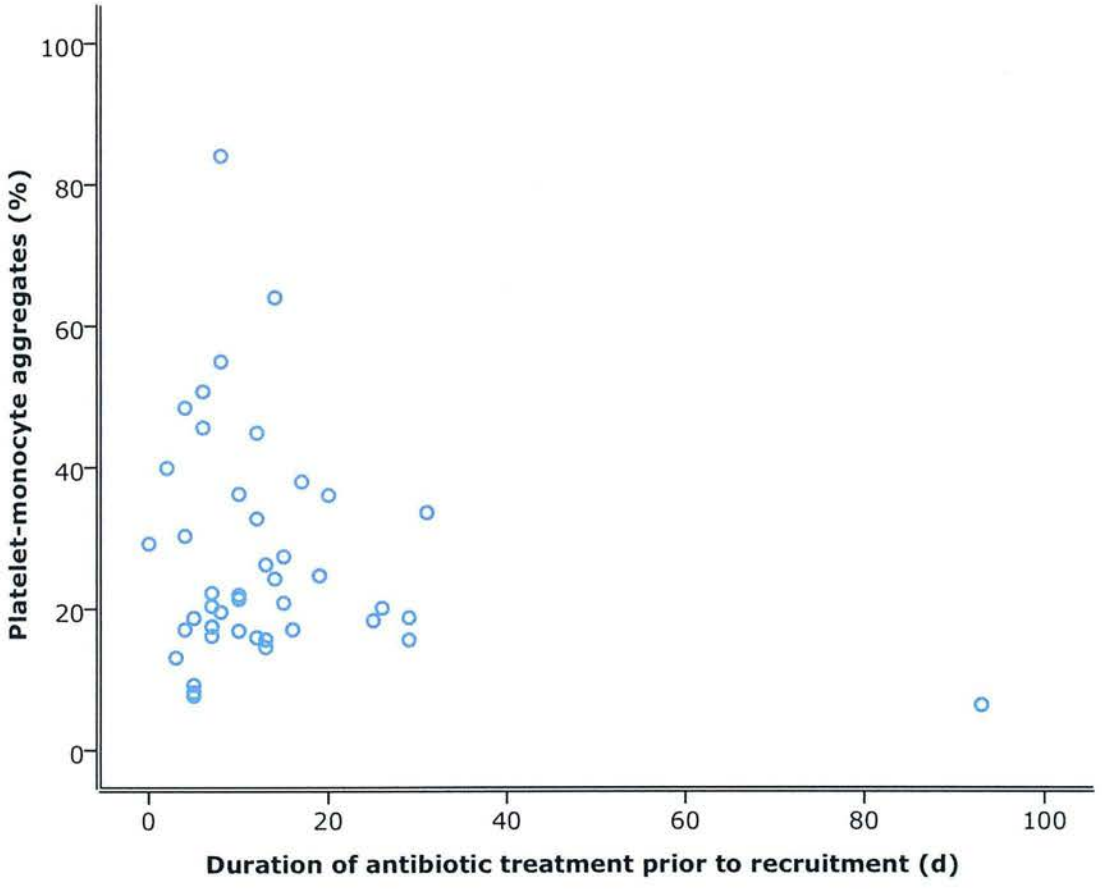

Figure 4.4. Scattergram of duration of antibiotic treatment prior to subject recruitment and platelet-monocyte aggregates in 44 patients with infective endocarditis. P=0.049, R^2=0.089.

4.3.3 Increased platelet activation is associated with need for valve replacement in infective endocarditis

The association of platelet activation with outcome in infective endocarditis was determined in the current study population (Table 4.9). Increased PMA was associated with adverse outcome as determined by the development of heart failure (P=0.081, Table 4.9) and need for valve replacement in infective endocarditis (P=0.003, Table 4.9). However, patients were variably recruited pre- and post-operatively and no assumptions could be made as to whether this was a predictive relationship.

There were no correlations between PMA or platelet P-selectin expression and development of vegetations, vegetation size or mobility, embolic events, renal failure, multi-organ failure or death when analysed individually (P>0.05 for all, Table 4.9). Analysis may have been limited by the modest study population and adverse outcome was therefore defined as the composite clinical end-point of embolism, heart failure, need for surgery and mortality. However, platelet activation did not correlate with development of the composite clinical end-point in patients with infective endocarditis (P=0.335 for PMA and P=0.899 for platelet P-selectin expression, Table 4.9).

Variables that significantly correlated with platelet activation on univariate analysis were further analysed by multivariate analysis. Presence of negative blood cultures and need for valve replacement remained significant for PMA, while coagulase-negative staphylococcal infection, intravenous drug abuse and treatment with aminoglycosides were correlated with platelet P-selectin expression on regression analysis (Table 4.10).

Table 4.9. Correlation between percentage platelet-monocyte aggregates (PMA), platelet P-selectin expression and clinical outcome in 44 patients with infective endocarditis.

Clinical outcome	Yes	No	P value
PMA			
Echocardiography			
Vegetation observed	26.3 ± 20.0	29.1 ± 11.5	0.382
Multiple vegetations	24.9 ± 15.7	27.4 ± 16.9	0.726
Valve destruction	27.0 ± 15.2	26.9 ± 17.3	0.883
Surgery	26.5 ± 15.6	27.6 ± 17.8	0.733
Replacement	30.5 ± 15.0	11.1 ± 4.8	0.003
Complications			
Heart failure	33.6 ± 20.4	24.2 ± 13.8	0.081
Acute renal failure	29.3 ± 20.3	25.4 ± 13.4	0.777
Multi-organ failure	25.5 ± 12.6	27.6 ± 17.9	0.885
Embolism	22.5 ± 10.8	30.0 ± 18.9	0.171
Relapse or recurrence	21.9 ± 5.6	27.6 ± 17.2	0.702
Death	23.1 ± 11.2	28.2 ± 17.6	0.475
Composite clinical end-point †	25.7 ± 14.3	30.4 ± 19.3	0.335

Clinical outcome	Yes	No	P value
P-selectin expression			
Echocardiography			
Vegetation observed	3.9 ± 3.4	3.9 ± 2.9	0.847
Multiple vegetations	2.8 ± 2.3	4.3 ± 3.5	0.477
Valve destruction	4.5 ± 3.0	3.5 ± 3.4	0.237
Surgery	3.7 ± 2.6	4.3 ± 4.1	0.978
Replacement	4.2 ± 2.7	1.7 ± 1.0	0.428
Complications			
Heart failure	4.9 ± 3.2	3.5 ± 3.3	0.193
Acute renal failure	3.2 ± 2.4	4.4 ± 3.7	0.390
Multi-organ failure	3.0 ± 2.3	4.3 ± 3.6	0.328
Embolism	3.4 ± 3.2	4.3 ± 3.3	0.289
Relapse or recurrence	4.5 ± 1.6	3.8 ± 3.4	0.411
Death	3.4 ± 2.9	4.1 ± 3.4	0.606
Composite clinical end-point †	3.8 ± 2.5	4.3 ± 4.7	0.899

Yes indicates that the clinical characteristic was present in the patient sub-group; no indicates that the clinical characteristic was absent. * Vegetation dimension and mobility data were available for 32 of the 34 patients with infective endocarditis † Composite clinical end-point, embolism, heart failure, need for surgery or mortality

Table 4.10. Multivariate analysis of the clinical features predictive of platelet-monocyte aggregate formation and platelet P-selectin expression in 44 patients with infective endocarditis.

Clinical variable	P value	R^2
Platelet-monocyte aggregates		
Valve replacement	0.008	0.265
Culture-negative	0.018	0.138
Coagulase-negative staphylococci	0.218	0.040
Platelet P-selectin expression		
Aminoglycoside treatment	0.003	0.308
Coagulase-negative staphylococci	0.037	0.128
Intravenous drug abuse	0.084	0.090
S. aureus	0.259	0.040
Venous thromboses	0.523	0.040

P was taken as <0.10.

4.4 Discussion

Activated platelets have been identified within the luminal layer of vegetations and are thought to arise following bacterium-induced activation (Fitzgerald et al., 2006a, Rouzet et al., 2008). However, platelets also contribute to immune defence (Yeaman, 1997), and it is not known whether platelets play a predominant role in thrombus formation or host immunity in infective endocarditis. This is the first study to use flow cytometry to determine platelet activation in patients with native and prosthetic valve infective endocarditis.

4.4.1 Study population

The age and gender demographics of recruited patients with infective endocarditis were similar to those observed in studies from different geographical regions (Heiro *et al.*, 2008, Murdoch *et al.*, 2009). There was large variation in time to patient presentation, consistent with known acute and sub-acute presentations of infective endocarditis (Bouza *et al.*, 2001). Prevalence of community-acquired disease, native valve involvement, history of previous infective endocarditis and intravenous drug abuse were similar to published studies (Bouza *et al.*, 2001, Heiro *et al.*, 2008, Murdoch *et al.*, 2009). However, rates of underlying congenital heart disease were almost twice those observed by other investigators, possibly due to regional variation (Alestig *et al.*, 2000, Fowler et al., 2005b). In some cases, there was a time delay from diagnosis or initiation of treatment to patient recruitment, largely due to the majority of patients being transferred from other hospitals for consideration of surgery. Approximately half of patients had a past history of arterial or venous thrombotic disease, which may place them at increased risk of infective endocarditis.

There has been recent controversy regarding the role of dental procedures as risk factors for infective endocarditis, resulting in changes in both national and international guidelines (Habib *et al.*, 2009, National Institute for Health and Clinical Excellence, 2008). In the current study, dental risk factors were identified in less than

7% of patients, similar to the levels observed by Fowler *et al* (Fowler et al., 2005b). However, it is unclear whether a history of recent dental work in patients with infective endocarditis is a causal relationship, or purely an association (National Institute for Health and Clinical Excellence, 2008).

In the current study, most cases of infective endocarditis were due to streptococci, conflicting with observations from recent large-scale studies (Cabell *et al.*, 2002, Heiro *et al.*, 2008, Moreillon & Que, 2004, Murdoch *et al.*, 2009). These discrepant findings may be due to the modest infective endocarditis cohort, the increased prevalence of *S. aureus* infective endocarditis in the USA, or due to the increased rates of congenital heart disease identified in this research. Of the 16 staphylococcal infective endocarditis cases, 6 were caused by coagulase-negative staphylococci, consistent with the recent increase in incidence of both native- and prosthetic-valve coagulase-negative staphylococcal infective endocarditis, which has been attributed to a rise in invasive procedures (Chu *et al.*, 2009, Chu *et al.*, 2008). In contrast to other published research, all of the *S. aureus* strains causing infective endocarditis in the current study were MSSA, which may be due to the small infective endocarditis study population and regional variation (Bouza et al., 2001, Cabell et al., 2002, Fowler et al., 2005b).

Cases affecting the mitral valve just outnumbered those affecting the aortic valve, similar to observations by Murdoch *et al* in their multicentre study on 2,781 patients with infective endocarditis (Murdoch *et al.*, 2009). Vegetations were present in three-quarters of cases, lower than frequencies observed in other studies, although abscesses or fistulae were present in a large number of patients in the current cohort (Murdoch *et al.*, 2009, Nadji *et al.*, 2005). There was evidence of cardiac conduction defects in approximately two-thirds of patients, higher than levels observed by other investigators (Bouza *et al.*, 2001, Murdoch *et al.*, 2009).

Approximately 60% of subjects required surgery for infective endocarditis, higher than rates observed in other series (Bouza *et al.*, 2001, Murdoch *et al.*, 2009). However, there was a referral bias as the ERI is a tertiary referral centre with a

cardiac surgery unit serving a large catchment area. Incidence of heart failure, acute renal failure and emboli were comparable to other studies, and mortality was within the range reported by other authors (Chu et al., 2004, Fowler et al., 2005b, Nadji et al., 2005).

4.4.2 Platelet activation in infective endocarditis

Platelet activation, measured as PMA and platelet P-selectin expression, was determined in 44 patients with infective endocarditis, 4 patients with bacteraemia and 30 age- and sex-matched healthy controls. Percentage PMA did not vary between patients with infective endocarditis and recruited controls, consistent with observations from a study of 76 patients with native valve infective endocarditis, where there were no associations between platelet activation, measured by β-thromboglobulin and platelet factor-4, and a diagnosis of infective endocarditis (Ileri *et al.*, 2003).

PMA is generally considered to be a more reliable marker of platelet activation than platelet surface receptor expression, presumably because platelets rapidly utilise surface-expressed factors such as P-selectin and GPIIb/IIIa in leucocyte-binding, reducing the number of sites available for antibody binding in flow cytometry (Michelson *et al.*, 2001, Spangenberg *et al.*, 1993). However, monocytes play a critical role in infective endocarditis and may influence the reliability of PMA as a marker of platelet activation. Specifically, monocytes are activated by staphylococci, phagocytose bacteria, release tissue factor, destroy valvular tissue and contribute to vegetation formation and propagation, all of which could modulate baseline PMA in infective endocarditis, regardless of platelet function (Chorianopoulos *et al.*, 2009, Durack, 1975, Veltrop *et al.*, 2000). Furthermore, there are large numbers of circulating monocytes in sepsis, which may result in lower percentages of platelet-bound monocytes than expected (Ogura *et al.*, 2001).

Although it has been reported that the formation of PMAs is dependent upon platelet and not leucocyte activation, it is not known how the presence of activated monocytes influences PMA formation (Ogura *et al.*, 2001, Russwurm *et al.*, 2002). In addition, agonist-stimulated platelets are themselves able to induce monocyte activation upon binding, but it is unclear whether this influences the course of sepsis (Bournazos *et al.*, 2008). Taking these factors into account, it may be that PMA is a less sensitive marker of platelet activation in infective endocarditis than other measures. A range of other platelet activation markers have been described, including CD40L, platelet factor-4 activated GPIIb/IIIa and platelet P-selectin expression (Harding *et al.*, 2004, Ileri *et al.*, 2003, Michelson, 1996, Michelson *et al.*, 2001). In the current study, platelet P-selectin expression was measured in addition to PMA.

A negative association was identified between P-selectin expression on circulating platelets and the development of infective endocarditis, consistent with observations by Yaguchi *et al* who identified reduced platelet activation and aggregation in patients with sepsis (Yaguchi *et al.*, 2004). However, most investigators have demonstrated increased platelet activation in sepsis, but their observations may not be comparable to the current study due to the use of different experimental techniques such as enzyme-linked immunosorbent assay (ELISA), or the use of different anticoagulants during venepuncture (Gawaz *et al.*, 1997, Gawaz *et al.*, 1995, Ogura *et al.*, 2001, Russwurm *et al.*, 2002).

Caution is required in the interpretation of results from this study, as analysis was limited by the study population size, large inter-subject variability in platelet activation and the presence of confounding factors. However, it can be hypothesised that platelet P-selectin expression was reduced in patients with infective endocarditis due to sequestration of activated platelets within vegetations or due to generalised platelet consumption, leaving fewer, less activated platelets to circulate in the bloodstream. Alternatively, reduced platelet activation may contribute to the pathogenesis of infective endocarditis by inhibition of platelet microbicidal protein (PMP) release from platelets, leading to enhanced bacterial growth and interactions

with thrombi in infective endocarditis (see Section 1.5.2). Antibiotic or anti-platelet treatment may have also contributed to reduced platelet activation in infective endocarditis (see Section 1.7).

4.4.3 Factors influencing platelet activation in infective endocarditis

In order to elucidate the possible reasons for reduced platelet activation in the infective endocarditis patient cohort, univariate and multivariate analyses of the correlations between PMA and platelet P-selectin expression and clinical characteristics in recruited patients were performed. On univariate analysis, platelet surface P-selectin expression was raised in intravenous drug abusers, consistent with a previous study which identified increased platelet activation in subjects with a history of cocaine use (Heesch *et al.*, 2000). Furthermore, platelet activation was increased in patients with a history of venous thromboses, correlating with other studies have determined platelet activation in both the early and late stages of deep venous thrombosis and pulmonary embolism (Blann *et al.*, 2000, Chung *et al.*, 2007). However, the observed correlation between venous thromboembolism and platelet P-selectin expression in the current study was no longer significant on multivariate analysis, possibly due to linkage with intravenous drug abuse or causative organisms such as *S. aureus* or coagulase-negative staphylococci (Fowler et al., 2005b, Moreillon & Que, 2004).

Platelet activation appeared to be influenced by pathogen-related factors in infective endocarditis as levels varied with causative organism. In the current study, the presence of staphylococci, particularly coagulase-negative species in blood cultures, was associated with increased platelet activation, correlating with the increased propensity of these organisms to induce platelet aggregation *in vitro* in comparison to organisms such as streptococci or enterococci (Kerrigan et al., 2002, O'Brien et al., 2002a, Usui *et al.*, 1991a, Usui *et al.*, 1991b). The mechanisms of *S. aureus*-induced platelet aggregation following growth in nutrient broth have been identified (see Section 1.4.3.3.2), while the mechanisms contributing to coagulase-negative

staphylococcus-induced platelet aggregation are still being determined. *S. epidermidis* induces platelet aggregation via the MSCRAMM SdrG (Brennan *et al.*, 2009), while the mechanisms of platelet aggregation induced by *S. lugdunensis*, which has been implicated in aggressive forms of infective endocarditis, are yet to be determined, but may involve the fibrinogen-binding protein Fbl (Mitchell *et al.*, 2004). Treatment with aminoglycosides was associated with reduced platelet activation in the current study, possibly due to its use in the treatment of streptococcal, rather than staphylococcal infective endocarditis (Habib *et al.*, 2009). The presence of negative blood cultures was associated with increased platelet activation, but there were a small number of subjects in this group (n=2).

There were also some important negative associations. CRP stimulates platelet activation, particularly in the context of ischaemic heart disease (Danenberg *et al.*, 2007), but there was no correlation between CRP levels at the time of sampling and PMA or platelet P-selectin expression. Additionally, the presence of anti-platelet therapy in patients with infective endocarditis did not influence platelet activation. There were concerns that route of venepuncture may influence platelet activation levels as this has been previously described, but no such trend was observed in the current study (Rubens *et al.*, 1998). Presence of a prosthetic valve at time of recruitment did not correlate with platelet activation, and in contrast to previous work, there was no significant correlation betweens underlying valvular heart disease and platelet activation (Chen *et al.*, 2004, Chirkov *et al.*, 2002, Pareti *et al.*, 2000, Tse *et al.*, 1997). Time of subject recruitment was marginally associated with increased PMA however, this was not a linear relationship and could have been influenced by other factors such as antibiotic and anti-platelet therapy and causative organism.

4.4.4 Platelet activation and outcome in infective endocarditis

Considering that cardiac vegetations are thought to form as a direct result of platelet aggregation (Bashore et al., 2006, Fitzgerald et al., 2006a), it was hypothesised that

patients with vegetations would have increased platelet activation. However, no correlation was observed between the presence of a vegetation, vegetation size or mobility and platelet activation, consistent with findings by Ileri *et al* (Ileri *et al.*, 2003). A possible explanation for these results is that vegetations are dynamic and undergo continuous change. For example, larger vegetations may fragment at any time such that a vegetation may appear small immediately after major embolisation has occurred (Ileri *et al.*, 2003). It is well established that the risk of embolisation is proportional to vegetation size (Di Salvo *et al.*, 2001, Fabri *et al.*, 2006, Thuny *et al.*, 2005, Tischler & Vaitkus, 1997).

One previous study has identified increased platelet activation in patients with infective endocarditis complicated by embolic phenomena, but the investigators excluded patients with prosthetic heart valves and those treated with anti-platelets or anticoagulants, which comprise a large proportion of patients present in a clinical setting (34% and 59% of the current study population respectively) (Ileri *et al.*, 2003). There was no correlation between platelet activation and development of emboli in the current study, possibly due to patient treatment with anti-platelets, anticoagulants and antibiotics prior to blood sampling.

Unfractionated heparin and warfarin increase platelet activation (Harding *et al.*, 2006a, Mieszczak & Winther, 1996, Wahba *et al.*, 1996), while clopidogrel, a platelet ADP antagonist, reduces PMA (Harding *et al.*, 2006b). However, detailed analyses of the patient cohort revealed no differences in platelet activation with anti-platelet or anticoagulant therapy in the current study, but such conclusions may be invalid due to the limited population size.

Need for valve replacement was associated with increased platelet activation in the infective endocarditis study population. However, the validity of this relationship is questionable as patients were recruited at various time-points during the disease process. It is feasible that increased platelet activation was associated with the underlying complication necessitating surgery (for example uncontrolled sepsis or valve degeneration), rather than valve replacement itself. Alternatively, temporary

rises in platelet activation during the early post-operative period may have confounded the issue (Goldsmith *et al.*, 2001, Leguyader *et al.*, 2006). Consistent recruitment of patients at defined intervals pre- or post-operatively would have enabled accurate determination of the predictive role of platelet activation in patients requiring valve replacement, and should be explored in future studies.

Platelet activation is thought to contribute to the development of DIC and multi-organ failure in patients with sepsis, and patients with organ failure may therefore be expected to have increased platelet activation levels (Gawaz *et al.*, 1997). However, PMA is paradoxically lower in septic patients with multi-organ failure and death, presumably due to peripheral sequestration of platelets (Gawaz *et al.*, 1995, Sunakawa *et al.*, 1988). In the current study, there was a trend towards increased percentage PMA in patients with heart failure but no associations with multi-organ failure, mortality or the composite clinical end-point of embolism, heart failure, need for surgery and mortality. Similarly, Ileri *et al* did not identify correlations between platelet activation and mortality (Ileri *et al.*, 2003).

4.4.5 Conclusions

In summary, this study is the first to demonstrate a link between platelet activation and disease development in infective endocarditis, as platelet P-selectin expression was reduced in patients with infective endocarditis in comparison to healthy volunteers. The low platelet P-selectin expression observed in patients with infective endocarditis may be due to platelet sequestration within vegetations or due to the role of platelets in immune defence, which should be analysed further by measuring PMP levels in patients with bacteraemia and infective endocarditis in comparison to healthy subjects (Dankert *et al.*, 2001, Tang *et al.*, 2002).

This research was severely limited by the modest study populations in the infective endocarditis and control groups, and by the variable timing of subject recruitment and blood sampling. Ideally, this study should have characterised the temporal

variation in platelet activation during the course of infective endocarditis in relation to factors such as antibiotic therapy, surgery, vegetation size and development of embolic complications, with correction for confounding factors such as causative organism, anti-platelet and anticoagulant treatment. Such methodological modifications should be incorporated in future studies, which should also aim to analyse platelet activation in large numbers of subjects, including controls with other causes of bacteraemia and underlying valvular heart disease or intravenous drug abuse without evidence of infective endocarditis, in order to determine the predictive role of PMA and platelet P-selectin expression in this condition.

CHAPTER 5

DETERMINATION OF THE INFLUENCE OF PLATELET RECEPTOR POLYMORPHISMS ON *S. AUREUS*-INDUCED PLATELET AGGREGATION AND CLINICAL OUTCOME IN INFECTIVE ENDOCARDITIS

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5.1 Introduction

Previous studies have observed host variation in susceptibility to bacterium-induced platelet aggregation *in vitro*. For example, Miajlovic *et al* found that 1 out of 3 donors had attenuated *S. aureus*-induced platelet aggregation, but did not elucidate the underlying mechanisms contributing to this (Miajlovic *et al.*, 2007). McNicol *et al* identified donor variation in response to *S. sanguinis*-induced platelet aggregation, which could not be fully explained by variation in IgG titres (McNicol *et al.*, 2006). Considering the role of specific platelet receptors in bacterium-induced platelet aggregation, it is feasible that platelet receptor polymorphisms influence host susceptibility to platelet aggregation and clinical outcome in infective endocarditis.

Genetic risk factors have previously been identified for infectious and inflammatory diseases. For example, heterozygosity for the factor V Leiden mutation has been associated with increased risk of urinary tract infections in a Danish population (Benfield *et al.*, 2005). Mannan-binding lectin-2 polymorphisms have been associated with increased risk of carditis in patients with rheumatic fever (Schafranski *et al.*, 2008). Furthermore, a number of studies have identified genetic risk factors for infections caused by *Streptococcus pneumoniae* and *Neisseria meningitidis* (Brouwer *et al.*, 2009).

Following the success of the Human Genome Project, the single nucleotide polymorphism (SNP) consortium was founded, with the aim of discovering novel SNPs contributing to human disease, thus potentially identifying individuals that would benefit most from tailored treatment or prophylaxis (Topol, 2008). Although numerous genome-wide association studies have focused on cardiovascular diseases such as MI or atrial fibrillation, none have investigated genetic predispositions to infective endocarditis (Topol, 2008).

Bacterium-platelet adhesion, activation and aggregation contribute to vegetation formation and the pathogenesis of infective endocarditis (see Section 1.4.3.3). In

addition, platelet receptor polymorphisms influence pharmacological agonist-induced platelet activation and aggregation, as well as the development of vascular and immune disease (see Section 1.6). However, the effects of polymorphic platelet receptors on bacterium-platelet interactions and outcome in infective endocarditis are unknown. Using a combination of flow cytometry and platelet aggregometry, the influence of platelet receptor polymorphisms on *S. aureus*-platelet adhesion, activation and aggregation was examined in the current study. The effects of platelet receptor genotype on susceptibility to and clinical outcome in infective endocarditis were also determined.

5.2 Methods

Platelet receptor genotype was determined for 164 healthy volunteers and 44 patients with infective endocarditis (see Sections 2.1 and 4.3.1). Venepuncture was performed on all subjects to obtain plasma for determination of platelet receptor polymorphisms as outlined in Sections 2.4 and 2.5.

S. aureus-induced aggregation of platelets in platelet-rich plasma (PRP) and washed platelet preparations was analysed by performing platelet aggregometry using blood from 88 and 31 healthy volunteers, respectively (see Section 2.11), selected on the basis of their platelet receptor genotype. Platelets were processed as described in Section 2.6. *S. aureus* strains Newman, 207 and 209 grown to stationary and mid-exponential phase in BHI were selected as agonists for aggregation studies and processed as described in Sections 2.8 and 2.9. The pharmacological agonists SFLLRN-NH_2, ADP and ristocetin were used as positive controls. *S. aureus*-platelet adhesion, baseline and *S. aureus*-induced platelet activation were determined using flow cytometry as outlined in Sections 2.9 and 2.10 using blood from 31 healthy subjects.

5.3 Results

5.3.1 Distribution of platelet receptor genotypes in healthy volunteers is similar to published studies

164 healthy volunteers were recruited for genotyping of platelet receptor polymorphisms. Results for the GPIb Kozak sequence polymorphism were obtained for 158 healthy volunteers (96%), GPIIIa platelet antigen ($PI^{A1/A2}$) and GPIb variable number of tandem repeat (VNTR) (Figure 5.1) polymorphisms for 160 subjects each (98%), and GPIb human platelet antigen-2 (HPA-2) and FcγRIIa H131R for 100% of subjects. Complete results for all five polymorphisms were obtained for 153 of the 164 healthy volunteers (93% total, 33% male, 30±10 years) and are outlined in Table 5.1. All polymorphisms were in Hardy-Weinberg equilibrium (data not shown).

Frequencies of platelet receptor polymorphisms in the healthy volunteer population were similar to those observed in published studies (Table 1.4) (Baker *et al.*, 2001, Laule *et al.*, 1999, Lehrnbecher *et al.*, 1999, Newman *et al.*, 1989, Jilma-Stohlawetz *et al.*, 2003). In the current study there was incomplete linkage disequilibrium between the GPIb VNTR and HPA-2 polymorphisms as 21 of 22 (95%) of VNTR A or B subjects carried an HPA-2b allele, while 23 out of 25 (92%) of HPA-2b-positive individuals carried a VNTR A or B allele, similar to frequencies observed by Ozelo *et al* (Ozelo *et al.*, 2004a).

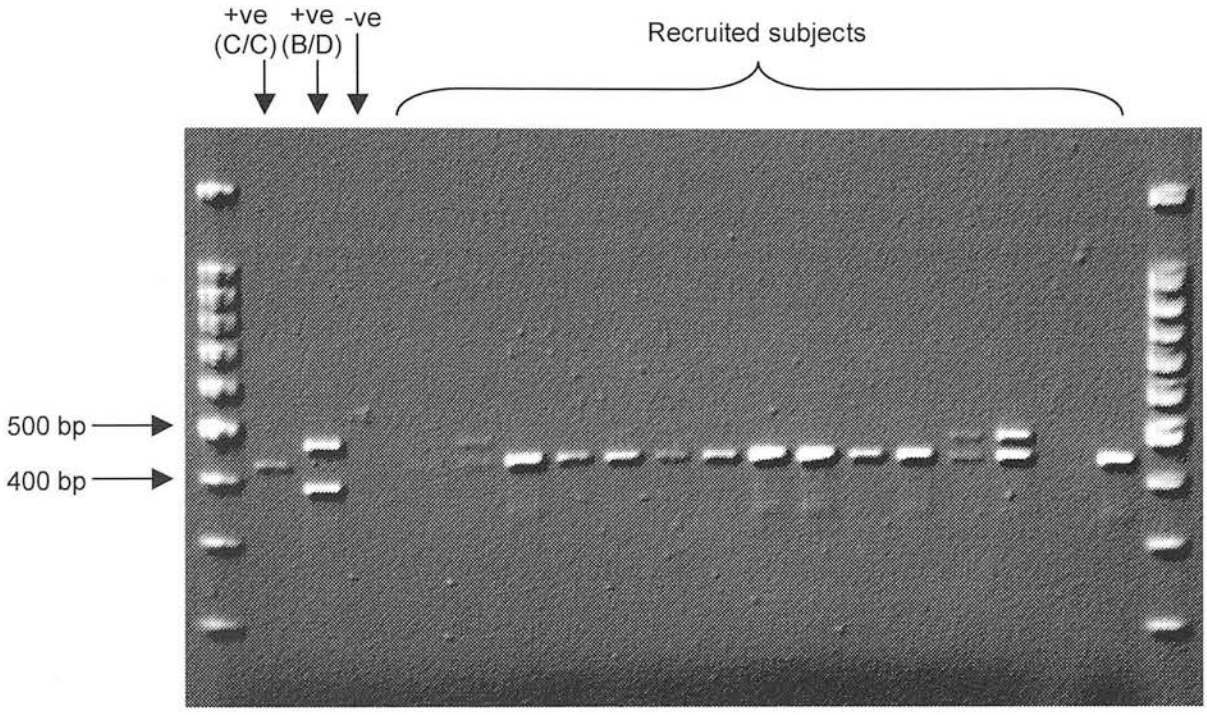


Figure 5.1. Representative agarose gel electrophoresis of PCR products used for determination of GPIb variable number of tandem repeat (VNTR) polymorphisms. Allele sizes are as follows: A (506 bp), B (467 bp), C (428 bp), D (389 bp) and E (250 bp). Lanes 1 and 2 are the positive controls with genotypes C/C and B/D, respectively, lane 3 is the negative control (distilled water) and the remainder are samples from recruited subjects.

Table 5.1. Frequencies of GPIIIa platelet antigen ($PI^{A1/A2}$), GPIb Kozak sequence, human platelet antigen (HPA)-2, variable number of tandem repeat (VNTR) and FcγRIIa H131R platelet receptor polymorphisms in 153 genotyped healthy volunteers and 88 subjects selected for platelet aggregometry.

Platelet receptor genotype	Healthy volunteers, n=153, (%)	Healthy volunteers n=88 *
GPIIIa $PI^{A1/A2}$		
$PI^{A1/A1}$	119 (78%)	58
$PI^{A1/A2}$	30 (20%)	26
$PI^{A2/A2}$	4 (2%)	4
GPIb Kozak sequence		
T/T	119 (78%)	57
T/C	34 (22%)	31
C/C	0 (0%)	0
GPIb HPA-2		
2a/2a	130 (85%)	67
2a/2b	22 (14%)	20
2b/2b	1 (1%)	1
GPIb VNTR		
D/D	2 (1%)	1
C/D	15 (10%)	11
C/C	114 (75%)	55
B/D	2 (1%)	2
B/C	19 (12%)	18
A/D	1 (1%)	1

Platelet receptor genotype	Healthy volunteers, n=153, (%)	Healthy volunteers n=88 *
FcγRIIa H131R		
R/R	47 (31%)	25
H/R	70 (46%)	40
H/H	36 (23%)	23

* Platelet receptor genotype for 88 of the 153 subjects selected for platelet aggregation studies. Subject selection was based on the presence of a variant allele.

5.3.2 Selection of healthy volunteers for platelet aggregation and flow cytometric studies

Healthy volunteers were selected for platelet aggregation studies based on the presence of a variant allele. Using these criteria, 99 healthy subjects were suitable for platelet aggregometry using PRP. However 11 (11%) had to be excluded because of long-term anti-platelet therapy (n=2), existing antibiotic therapy (n=1), loss to follow-up (n=5) and 3 were outliers as their results were greater than 3 standard deviations from the mean, leaving a study population of 88 healthy volunteers (40% male, 30±9 years, Table 5.1).

Additional studies were performed to determine whether the GPIIIa $PI^{A1/A2}$, GPIb Kozak sequence and FcγRIIa H131R polymorphisms influenced *S. aureus*-platelet adhesion, activation or solely aggregation. To exclude the effects of variation in plasma factors such as fibrinogen or IgG between different donors, platelet aggregometry was performed with washed platelets from representative subjects. Although 33 subjects were identified as being suitable for these studies, 2 of the 4 individuals with the $PI^{A1/A2}$ T/C genotype were lost to follow up, leaving a study population of 31 subjects (52% male, 33±9 years, Table 5.2).

Table 5.2. GPIIIa platelet antigen ($PI^{A1/A2}$), GPIb Kozak sequence and FcγRIIa H131R platelet receptor genotypes in 31 healthy volunteers selected to participate in studies determining the effect of platelet receptor polymorphisms on *S. aureus*-platelet adhesion, activation and aggregation of washed platelets.

Platelet receptor genotype	Healthy volunteers, n=31
GPIIIa $PI^{A1/A2}$	
$PI^{A1/A1}$	17
$PI^{A1/A2}$	10
$PI^{A2/A2}$	4
GPIb Kozak sequence	
T/T	19
T/C	12
C/C	0
FcγRIIa H131R	
R/R	8
H/R	16
H/H	7

5.3.3 Selection of *S. aureus* strains for platelet aggregation and flow cytometric studies

S. aureus strains Newman, 209 and 207 grown to stationary and mid-exponential phase in BHI were selected for *in vitro* assays, to enable determination of the effects of platelet receptor polymorphisms on platelet adhesion, activation and aggregation induced by strains that were rapid, medium and slow inducers of platelet aggregation, respectively (Figure 5.2). Note that *S. aureus* strain 207 grown to stationary phase only induced platelet aggregation within 25 min with 1 out of the 88 donors' PRP (Figure 5.2) and was therefore excluded from subsequent analyses. Furthermore, only 4 subjects' washed platelet preparations aggregated in response to strain 207 at the exponential phase of growth (data not shown). Pharmacological agonists ADP, ristocetin and SFLLRN-NH_2 were used as positive controls in aggregation studies and were specifically used at high concentrations to induce rapid and high percentage platelet aggregation (data not shown) (Lasne *et al.*, 1997).

Multivariate analysis using a general linear model could only be performed on data from PRP aggregation studies that fit a normal distribution. Lag time to aggregation induced by strain Newman grown to stationary and exponential phase and strain 209 grown to exponential phase could be transformed to fit a normal distribution. Percentage platelet aggregation induced by strain Newman at the stationary and exponential phases of growth and strain 209 grown to stationary phase also met the assumptions of normality. However, rate of aggregation could not be transformed to fit a normal distribution. Only the effects of two genotypes in combination could be analysed due to small study population sizes, particularly for the rare genotypes.

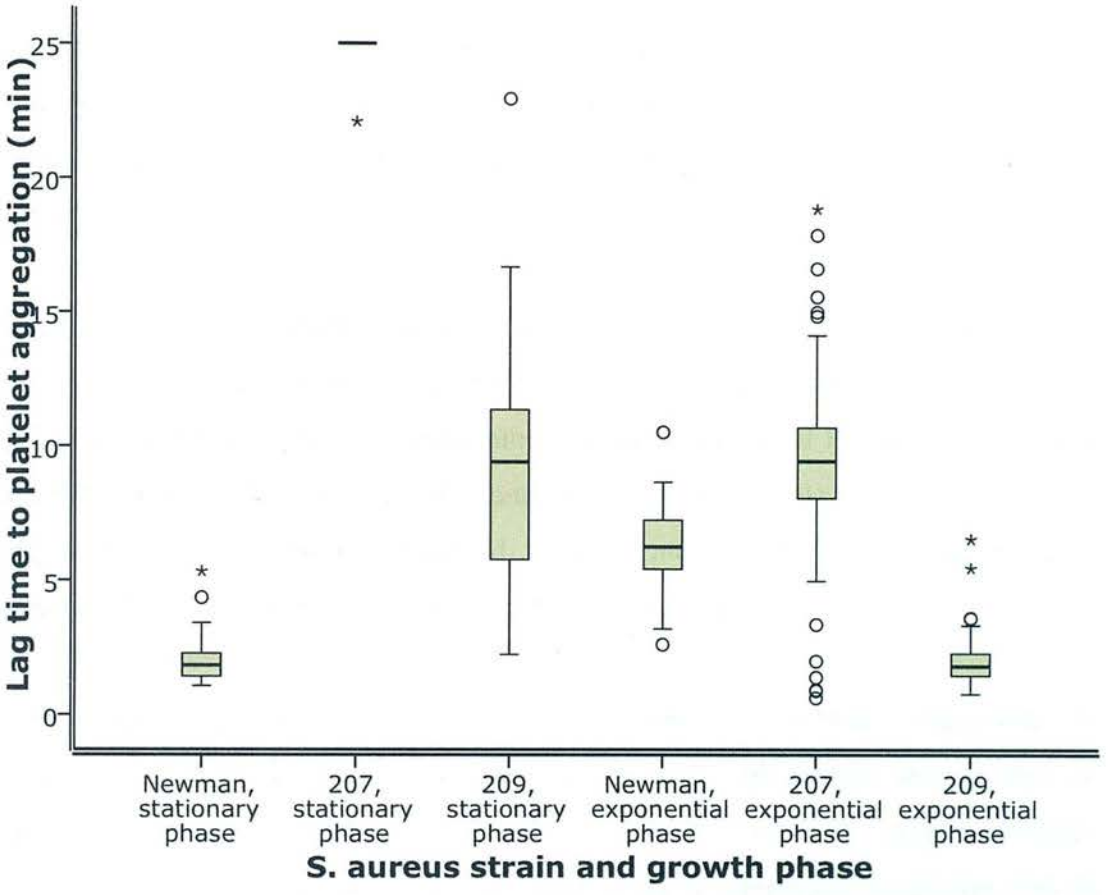


Figure 5.2. Distribution of lag time to platelet aggregation induced by *S. aureus* strains using platelets in platelet-rich plasma from 88 healthy volunteers. Circles and asterisks represent data points more than 2 and 3 standard deviations from the median, respectively.

S. aureus-platelet adhesion was determined by calculating the percentage of *S. aureus* cells bound to platelets and the percentage of platelets bound to *S. aureus* as outlined in Figure 5.3. Consistent with findings by Yeaman *et al*, there was heterogeneity in the ability of different *S. aureus* strains to bind platelets, possibly representing variation in MSCRAMM expression (data not shown) (Yeaman et al., 1992c).

Baseline percentage platelet activation was determined using whole blood platelet-monocyte aggregates (PMA) and platelet P-selectin expression, while *S. aureus*-induced platelet activation was determined by measurement of P-selectin expression in PRP. Both absolute (Figure 5.4A) and relative (Figure 5.4B) platelet activation in response to *S. aureus* were determined, the latter measured by subtraction of values obtained for vehicle alone from those obtained for each agonist.

Platelet activation induced by *S. aureus* did not approach levels associated with the positive control SFLLRN-NH_2, despite experimental modifications such as shaking platelet-*S. aureus* incubations, performing incubations at 37°C, using increased concentrations of fluorescent-conjugated antibodies or increasing incubation periods for the *S. aureus*-PRP mixture in the dark (data not shown). Most of the obtained relative values were negative, and absolute values were therefore used for analysis. Bacterium-induced platelet activation was greater for strain Newman, particularly at the stationary phase of growth as compared to other strains, correlating with the ability of this strain to induced rapid platelet aggregation at both growth phases (Figure 5.2).

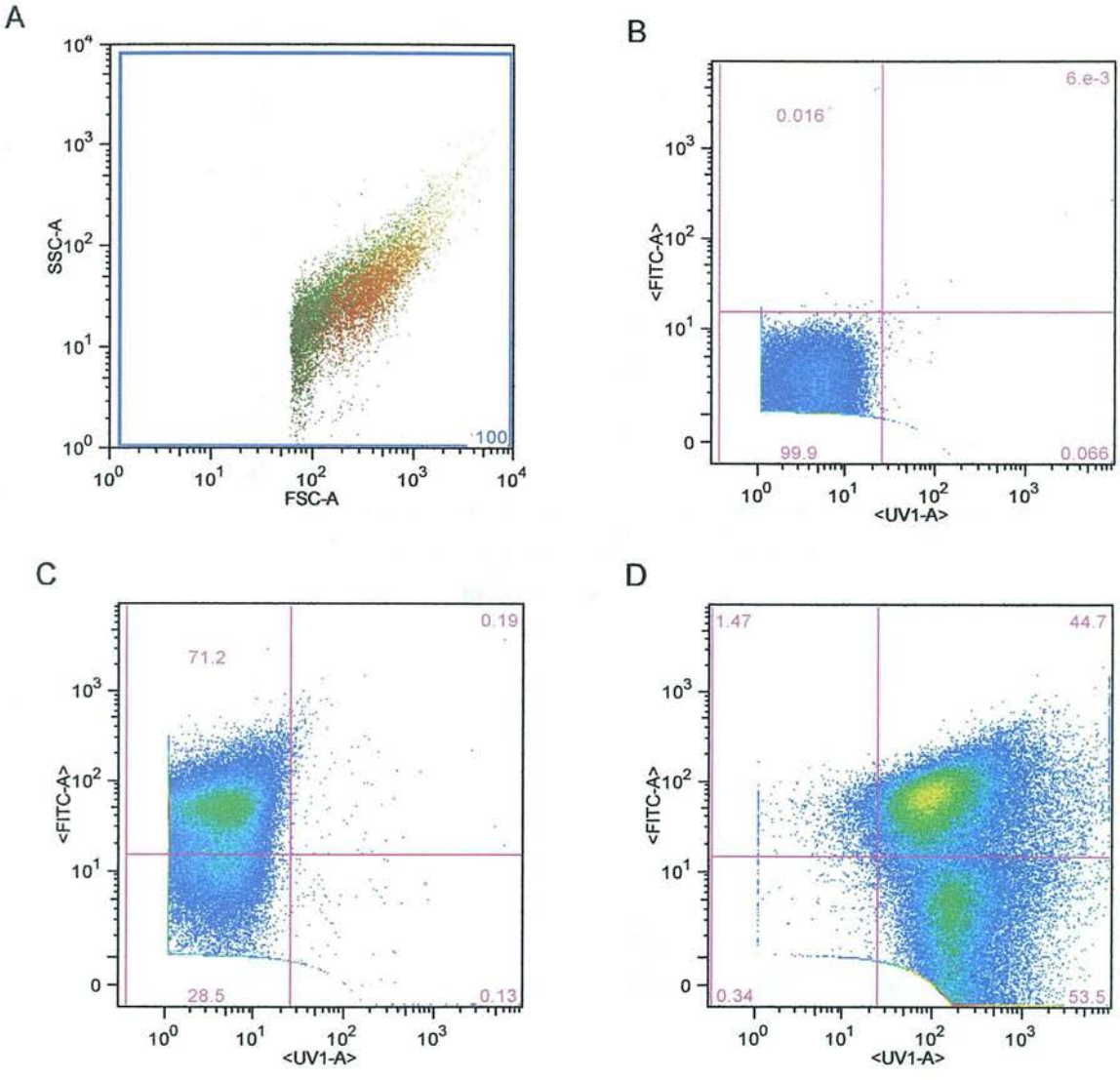


Figure 5.3. Flow-cytometric analysis of *S. aureus*-platelet binding for strain Newman grown to stationary phase. Dot blots of forward scatter (FSC-A) against side scatter (SSC-A) were used to identify cell populations (A); *S. aureus* (red), platelets (green). Quadrant plots of unstained samples (B) and samples stained with anti-CD42a-FITC alone (representing platelets) (C) were used to align gates. Cells stained with both anti-CD42a-FITC and Hoechst 33342, (represented on axes labelled FITC and UV-1 respectively), represent platelets bound to *S. aureus* cells (D).

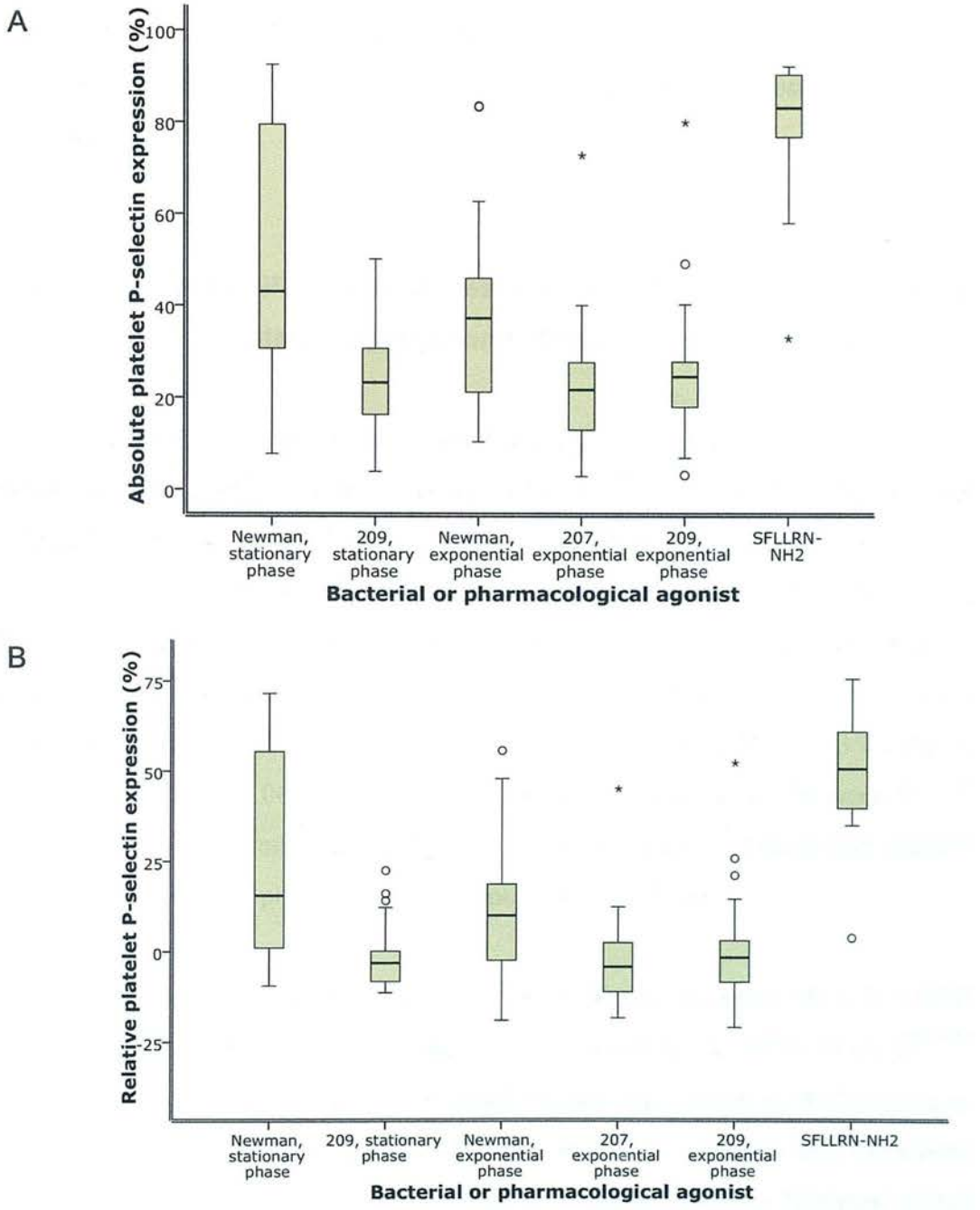


Figure 5.4. Data distribution for absolute (A) and relative (B) *S. aureus*-platelet activation, measured by platelet P-selectin expression in platelet-rich plasma from 31 healthy volunteers. Relative values were obtained by subtracting platelet P-selectin expression for vehicle alone (PBS for bacteria and 0.9% NaCl for SFLLRN-NH_2) from results obtained for each agonist. SFLLRN-NH_2 was used as the positive control. Circles and asterisks represent data points more than 2 and 3 standard deviations from the median respectively.

5.3.4 Platelet receptor polymorphisms influence *S. aureus*-induced platelet activation and aggregation, but not *S. aureus*-platelet adhesion

5.3.4.1 The GPIIIa PI^{A1} allele is associated with increased *S. aureus*-induced platelet aggregation independent of plasma factors

In studies using PRP, lag time to platelet aggregation induced by *S. aureus* was shorter for GPIIIa PI^{A1} carriers as opposed to $PI^{A2/A2}$ platelets, but this was not statistically significant ($PI^{A1/A1}$ 6.09 ± 1.40 min vs. $PI^{A1/A2}$ 6.63 ± 1.20 min, $PI^{A2/A2}$ 6.92 ± 1.03 min for strain Newman grown to exponential phase, P=0.060, Table S5.1). Additionally, there was a trend towards increased percentage platelet aggregation in response to strain 209 grown to stationary phase in platelets expressing GPIIIa $PI^{A1/A1}$ compared to PI^{A2} ($PI^{A1/A1}$ 68 ± 9 % vs. $PI^{A1/A2}$ 63 ± 10% vs. $PI^{A2/A2}$ 63 ± 33 %, P=0.060). There were no significant correlations between $PI^{A1/A2}$ genotype and rate of aggregation (Table S5.2), or maximal percentage platelet aggregation induced by pharmacological agonists (Table S5.3).

Using various combinations of GPIIIa $PI^{A1/A2}$, GPIb Kozak sequence, HPA-2, VNTR and FcγRIIa H131R polymorphisms, there was variation in the ability of the $PI^{A1/A2}$ polymorphism to influence *S. aureus*-induced platelet aggregation on multivariate as compared to univariate analysis. Specifically, the $PI^{A1/A1}$ genotype was associated with reduced lag time to platelet aggregation in response to strains Newman grown to stationary phase and 209 grown to mid-exponential phase (P=0.011 and 0.001 respectively, Figure 5.5), while it no longer influenced lag time to aggregation induced by strain Newman at the exponential phase of growth (P=0.147). This difference may be attributed to correction for other polymorphisms or the use of non-parametric tests for the analysis of univariate data and parametric tests for the analysis of multivariate data.

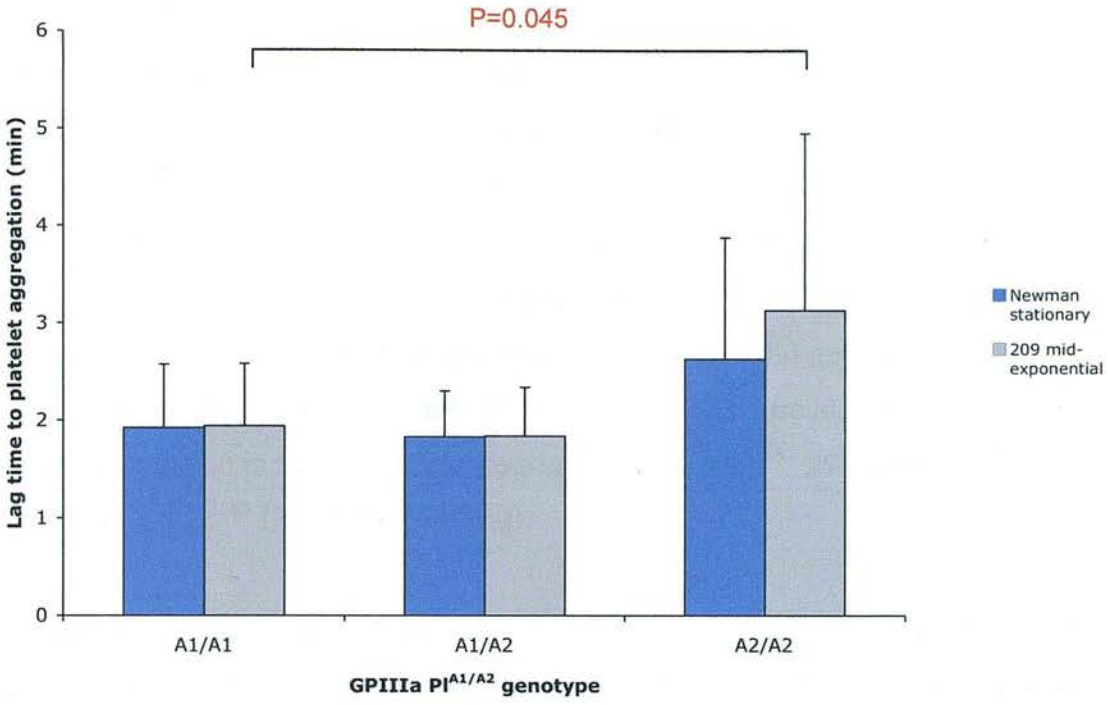


Figure 5.5. Effect of GPIIIa $PI^{A1/A2}$ genotype on lag time to platelet aggregation induced by *S. aureus* strain Newman grown to stationary phase (blue, P=0.011) and strain 209 grown to mid-exponential phase (grey, P=0.001) on multivariate analysis when using platelets in platelet-rich plasma from 88 subjects. Only results for the latter were significant on post-hoc analysis.

In studies using washed platelets, the Pl^{A1} allele was associated with reduced lag time to aggregation in response to strain Newman grown to stationary phase (P=0.034, Figure 5.6A). Conversely, platelets from subjects homozygous for the Pl^{A1} allele did not aggregate in response to strain 207 grown to exponential phase ($Pl^{A1/A1}$ 25 min vs. $Pl^{A1/A2}$ 19.24 ± 8.78 min vs. $Pl^{A2/A2}$ 22.93 ± 4.14 min, P=0.047, Table S5.4). Rate of aggregation was increased with the $Pl^{A1/A2}$ genotype (P=0.042, Figure 5.6B, Table S5.5). Percentage platelet aggregation did not vary with $Pl^{A1/A2}$ genotype for bacterial agonists (Table S5.6), but there was a trend towards increased platelet aggregation in Pl^{A1} carriers in response to ADP ($Pl^{A1/A1}$ 29 ± 20 % vs. $Pl^{A1/A2}$ 36 ± 17 % vs. $Pl^{A2/A2}$ 12 ± 16 %, P=0.062).

Baseline platelet activation did not vary with $Pl^{A1/A2}$ genotype (PMA: $Pl^{A1/A1}$ 27.1 ± 9.2 % vs. $Pl^{A1/A2}$ 24.4 ± 15.1 % vs. $Pl^{A2/A2}$ 28.5 ± 8.3 %, P=0.457; platelet P-selectin expression: $Pl^{A1/A1}$ 4.1 ± 2.0 % vs. $Pl^{A1/A2}$ 3.0 ± 1.4 % vs. $Pl^{A2/A2}$ 3.3 ± 1.0 %, P=0.296). There were no significant correlations between GPIIIa $Pl^{A1/A2}$ genotype and *S. aureus*-platelet binding or *S. aureus*-induced platelet activation (Tables S5.7-S5.9).

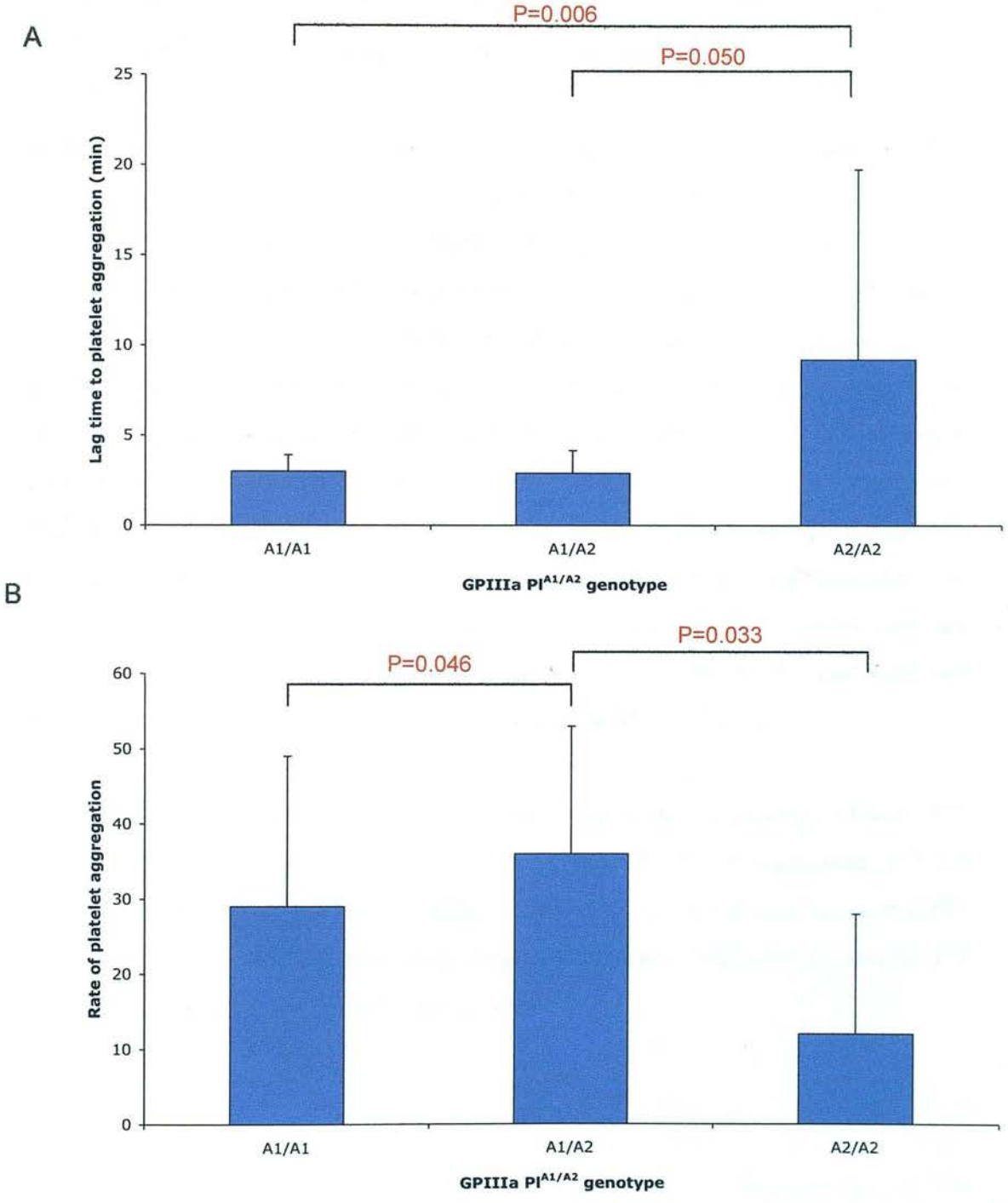


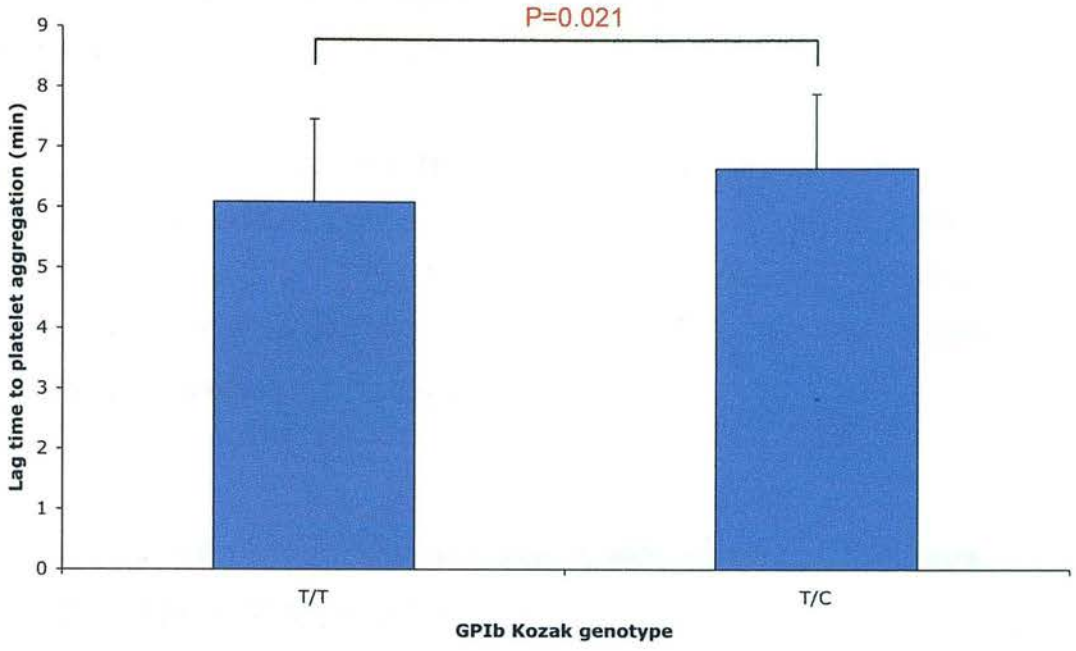
Figure 5.6. Effect of GPIIIa $PI^{A1/A2}$ genotype on lag time to platelet aggregation induced by *S. aureus* strain Newman grown to stationary phase (A) and rate of platelet aggregation induced by *S. aureus* strain 209 grown to stationary phase (B) Studies were performed using washed platelets from 31 subjects. P values were derived from post-hoc analysis.

5.3.4.2 The GPIb Kozak sequence polymorphism influences *S. aureus*-induced platelet aggregation in PRP

Results for variation in lag time, rate and percentage aggregation of platelets in PRP by GPIb Kozak sequence genotype are outlined in Tables S5.10–S5.12. Lag time to platelet aggregation was shorter for the Kozak sequence T/T as compared to the T/C variant in response to strain Newman grown to exponential phase (P=0.021, Figure 5.7A). There was no association between Kozak sequence genotype and rate of platelet aggregation (Table S5.11), while percentage platelet aggregation was significantly increased in T/C platelets in response to strain 209 at the stationary phase of growth (P=0.033, Figure 5.7B). The GPIb Kozak sequence polymorphism had no influence on platelet aggregation induced by pharmacological agonists (Tables S5.11-S5.12). The effects of the GPIb Kozak sequence polymorphism on lag time and maximal percentage platelet aggregation induced by *S. aureus* were not present on multivariate analysis (P=0.196 and P=0.056, respectively) and were not reproduced in studies using washed platelets (Tables S5.13-S5.15).

Baseline platelet activation did not vary with Kozak sequence genotype (PMA: T/T 26.3 ± 12.2 % vs. T/C 26.6 ± 9.6 %, P=0.685; platelet P-selectin expression: T/T 3.6 ± 2.1 % vs. T/C 3.6 ± 1.2 %, P=0.478). There were no correlations between GPIb Kozak sequence genotype and *S. aureus*-platelet adhesion (Tables S5.16 and S5.17) or *S. aureus*-induced platelet activation (Table S5.18).

A



B

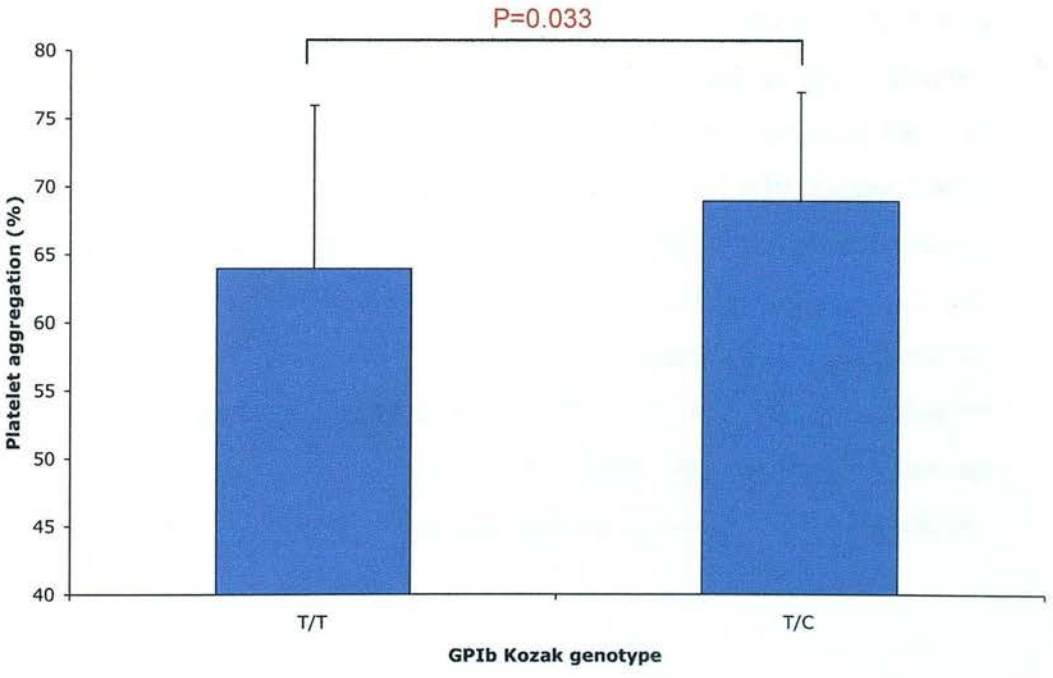


Figure 5.7. Effect of GPIb Kozak sequence genotype on lag time to platelet aggregation induced by *S. aureus* strain Newman grown to exponential phase (A) and percentage platelet aggregation induced by *S. aureus* strain 209 grown to stationary phase (B) using platelets in platelet-rich plasma from 88 healthy volunteers.

5.3.4.3 The GPIb HPA-2 polymorphism does not influence *S. aureus*-induced platelet aggregation

There were no correlations between HPA-2 genotype and lag time, rate and percentage platelet aggregation induced by *S. aureus* strains or pharmacological agonists in studies using PRP (Table S5.19-S5.21). Consequently, aggregation studies using washed platelets and flow cytometric analysis of bacterium-platelet adhesion and activation were not performed.

5.3.4.4 The GPIb VNTR D allele is associated with increased *S. aureus*-induced platelet aggregation

There were no significant associations between VNTR genotype and lag time or rate of platelet aggregation in studies using PRP (Tables S5.22 and S5.23). Maximal percentage platelet aggregation was increased for platelets heterozygous for a D allele in response to strain 209 grown to stationary phase (P=0.030, Figure 5.8A), which remained significant on multivariate analysis (P=0.007), and a similar trend was observed with most other strains (Table S5.24). Percentage aggregation was also significantly increased for B/D, C/C and C/D, as compared to B/C platelets, for the pharmacological agonist ristocetin (Figure 5.8B, P=0.016). As a number of individuals carrying the D allele were lost to follow up, studies determining bacterium-platelet adhesion, activation and aggregation of washed platelets were not performed.

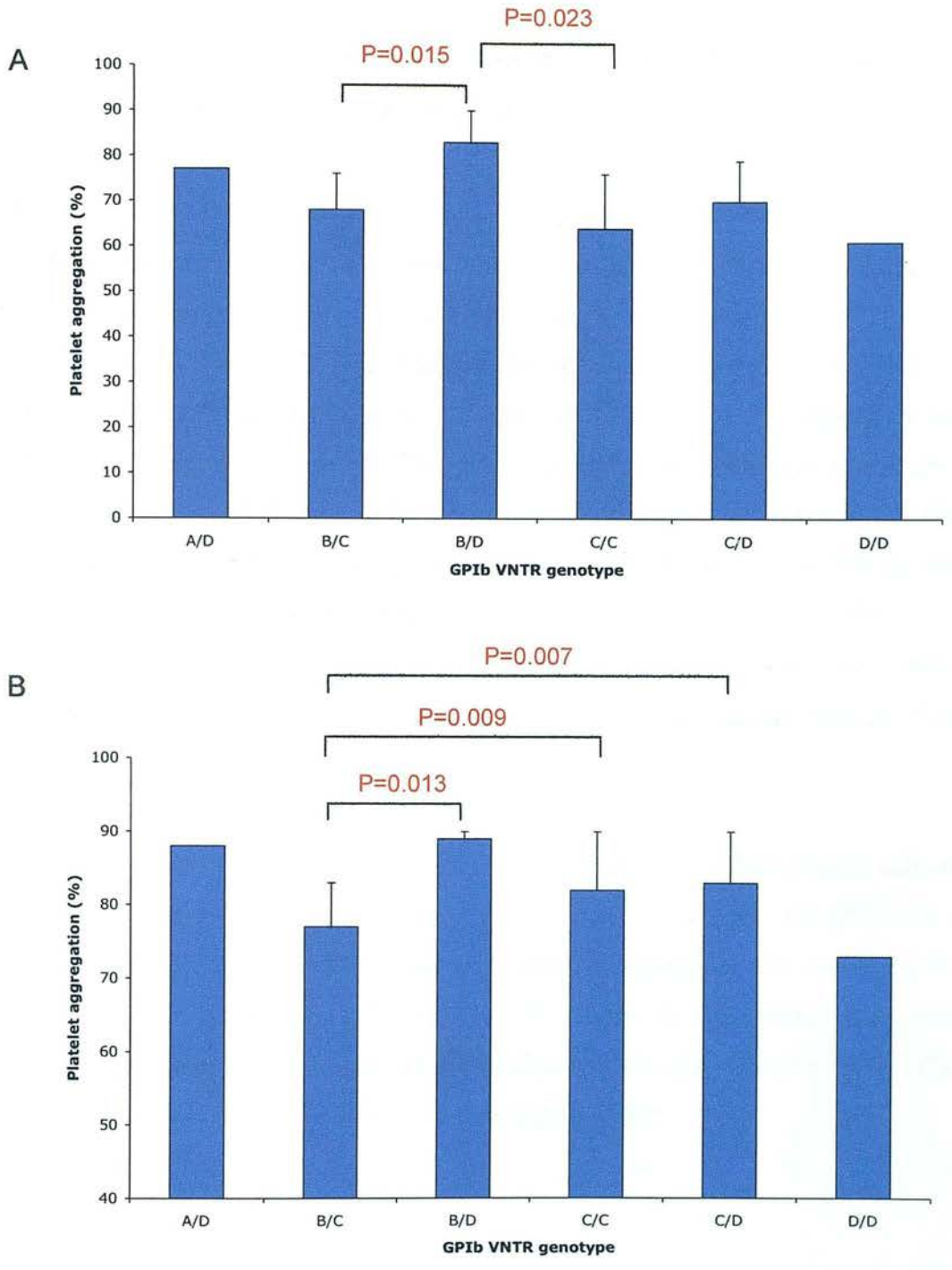


Figure 5.8. Effect of GPIb variable number of tandem repeat (VNTR) genotype on percentage platelet aggregation induced by *S. aureus* strain 209 grown to stationary phase (A) and percentage platelet aggregation induced by ristocetin (B) when using platelet-rich plasma from 88 healthy volunteers.
A/D and D/D genotypes were present in platelets from one individual each.

5.3.4.5 The FcγRIIa H131 allele is associated with increased *S. aureus*-induced platelet aggregation

In studies using PRP, lag time to platelet aggregation was reduced with FcγRIIa H/H platelets and this approached significance for strain Newman at the exponential phase of growth (H/R 6.67 ± 1.29 min vs. H/H 5.90 ± 1.61 min vs. R/R 5.92 ± 1.07 min, P=0.077, Table S5.25). In addition, rate of platelet aggregation was increased for H131 platelets in response to *S. aureus* strain 209 grown to stationary phase (P=0.038, Figure 5.9A, Table S5.26). H131 platelets were associated with increased percentage aggregation induced by strain 209 at the exponential phase of growth on univariate analysis (P=0.043, Figure 5.9B, Table S5.27), and strain 209 grown to stationary phase on multivariate analysis (H/H 68 ± 9% vs. H/R 67 ± 10 % vs. R/R 64 ± 14%, P=0.017). The effects of the FcγRIIa H131 allele on *S. aureus*-induced platelet aggregation were not reproduced in studies using washed platelets (Tables S5.28-S5.30).

S. aureus-platelet adhesion (Tables S5.31 and S5.32) and baseline platelet activation did not vary with H131R genotype (PMA: H/H 28.5 ± 11.3 % vs. H/R 26.9 ± 12.5 % vs. R/R 24.2 ± 8.8 %, P=0.719; platelet P-selectin expression: H/H 4.4 ± 1.1 % vs. H/R 3.5 ± 2.2 % vs. R/R 3.4 ± 1.2 %, P=0.198). However, there was a trend towards increased *S. aureus*-induced platelet activation with H/H platelets (H/H 49 ± 24% vs. H/R 38 ± 16% vs. R/R 25 ± 15%, P=0.059, Table S5.33).

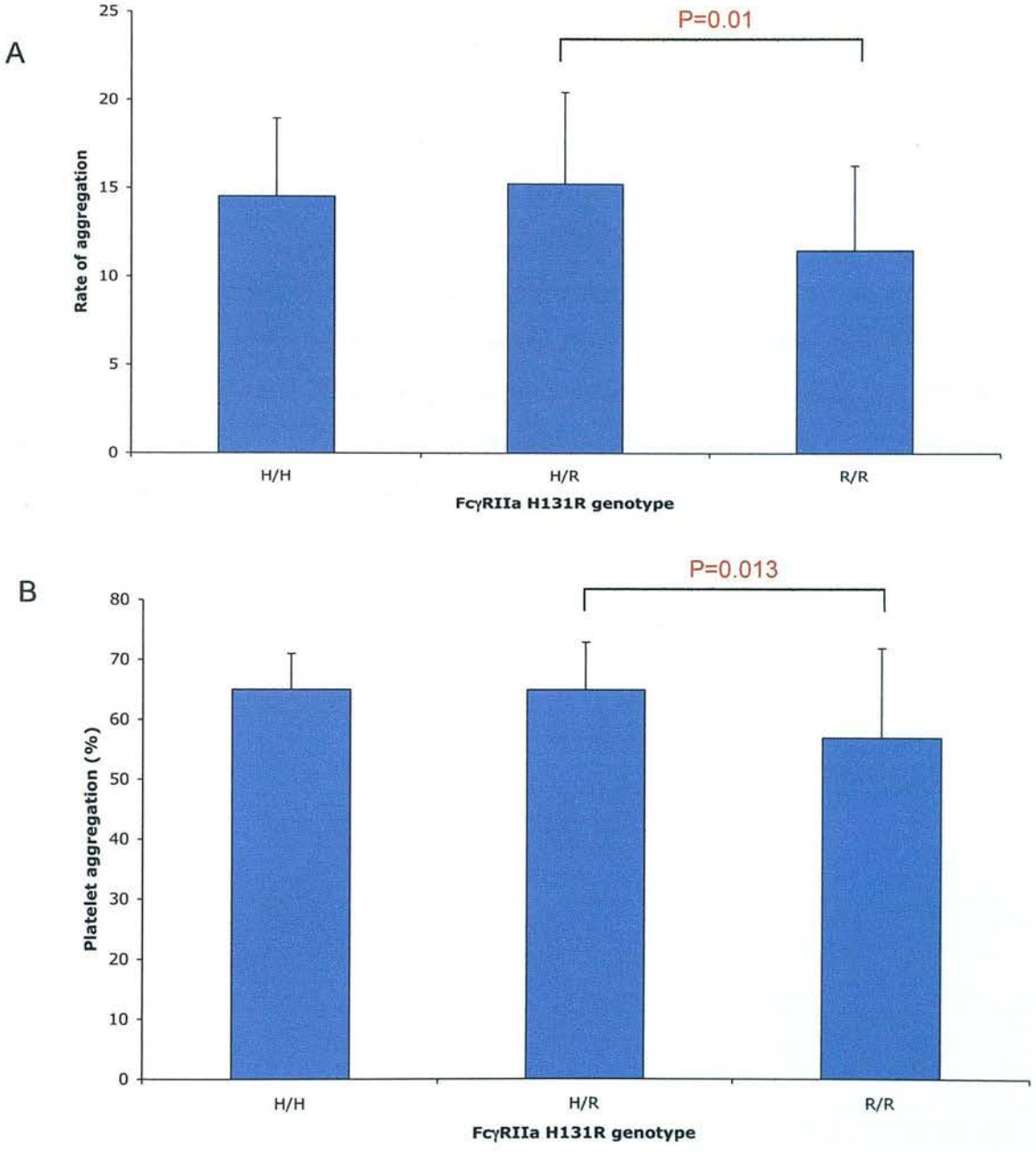


Figure 5.9. Effect of FcγRIIa H131R genotype on rate of platelet aggregation induced by *S. aureus* strain 209 at the stationary phase of growth (A) and percentage platelet aggregation induced by *S. aureus* strain 209 at the exponential phase of growth (B) using platelet-rich plasma from 88 healthy volunteers. P values were obtained from post-hoc analysis.

5.3.4.6 GPIIIa $PI^{A1/A2}$ and GPIb Kozak sequence polymorphisms have an interactive effect on *S. aureus*-induced platelet aggregation

Regression analysis revealed an interactive effect of GPIIIa $PI^{A1/A2}$ and GPIb Kozak sequence genotypes on lag time to platelet aggregation (Figure 5.10). In addition to PI^{A1} homozygotes, aggregation was more rapid with $PI^{A1/A2}$ T/T and $PI^{A2/A2}$ T/C platelets in response to strain Newman at the stationary phase of growth (P=0.042) and strain 209 grown to exponential phase (P=0.012). However, the ability of GPIIIa $PI^{A1/A2}$ and GPIb Kozak sequence to collectively influence *S. aureus*-platelet adhesion, activation and aggregation of washed platelets could not be determined due to the number of subjects recruited for these studies.

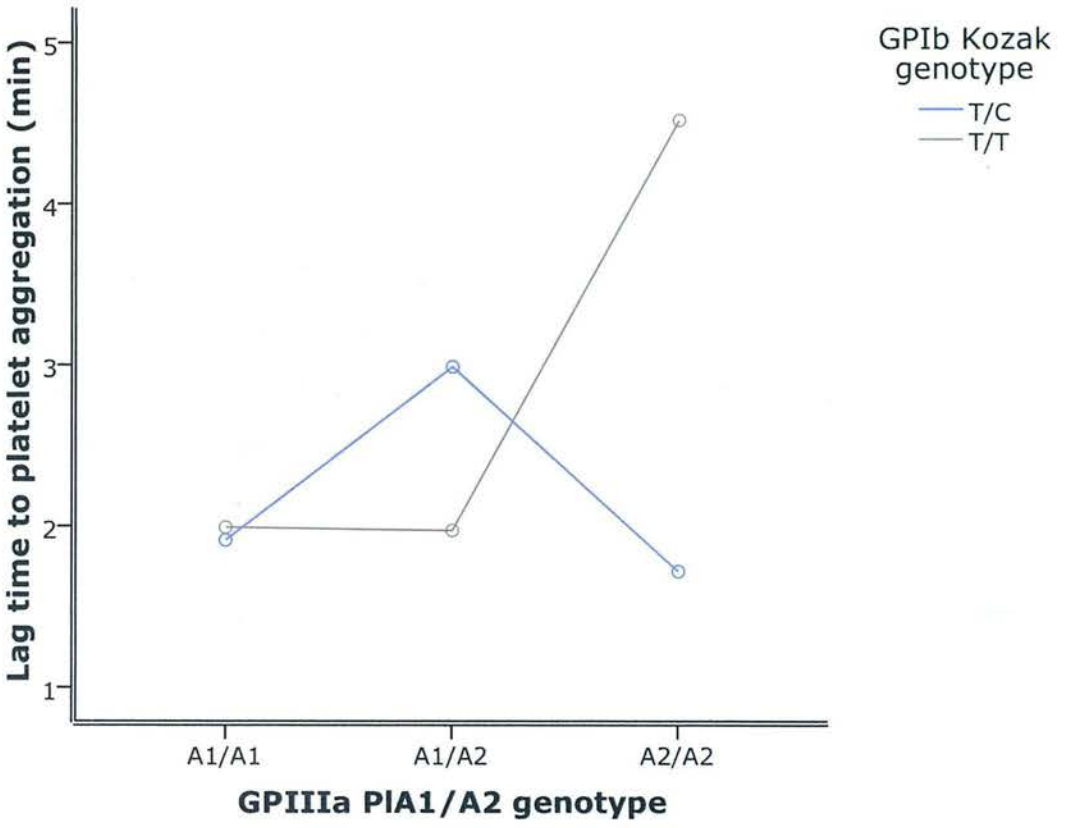


Figure 5.10. Interactive effects of the GPIIIa $PI^{A1/A2}$ and GPIb Kozak sequence genotypes on lag time to platelet aggregation induced by *S. aureus* strain 209 grown to mid-exponential phase when using platelet-rich plasma from 88 healthy volunteers.

5.3.5 Platelet receptor polymorphisms are associated with adverse outcome in infective endocarditis

In order to determine the correlation between polymorphic platelet receptors and disease severity in infective endocarditis, platelet receptor genotypes were determined for 44 patients with infective endocarditis (see Section 4.3.1). Results for GPIIIa $Pl^{A1/A2}$ and FcγRIIa HI31R were obtained for 41 subjects each (93%), GPIb HPA-2 for 40 subjects (91%), Kozak sequence for 39 subjects (89%) and VNTR for 38 patients (86%). Complete results were obtained for 35 subjects (80%). There was no difference in the distribution of these polymorphisms in comparison to a healthy population (Figure 5.11).

The association of each of these polymorphisms with the severity of infective endocarditis was determined.

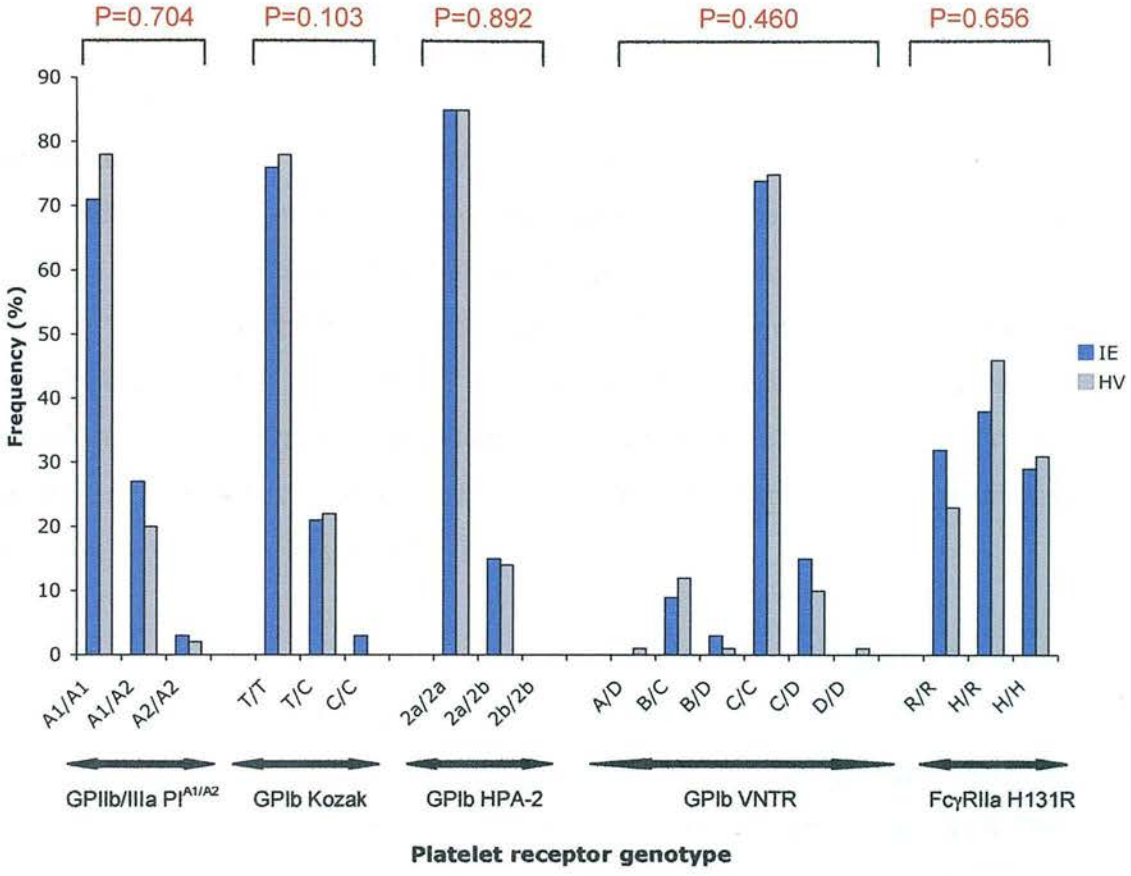


Figure 5.11. Distribution of GPIIIa $PI^{A1/A2}$, GPIb Kozak sequence, human platelet antigen-2 (HPA-2), variable number of tandem repeat (VNTR) and FcγRIIa H131R platelet receptor polymorphisms in patients with infective endocarditis (IE, n=44) as compared to healthy volunteers (HV) for whom a complete platelet receptor genotype profile was available (n=153).

5.3.5.1 The GPIIIa PI^{A1} allele is associated with increased platelet activation and infective endocarditis causation by staphylococci and streptococci

Consistent with results from *in vitro* studies, platelet activation measured by both PMA and P-selectin expression was significantly increased for $PI^{A1/A1}$ subjects compared to $PI^{A1/A2}$ subjects (P=0.001 and 0.026, respectively, Figure 5.12A). The one $PI^{A2/A2}$ patient had high PMA and platelet P-selectin expression levels of 84.0 % and 7.9 % respectively. The $PI^{A1/A1}$ genotype was associated with development of *Staphylococcus*- or *Streptococcus*-induced endocarditis, while the $PI^{A1/A2}$ variant was linked to endocarditis caused by *Enterococcus spp.* or other organisms detailed in Section 4.3.1 (P=0.004, Figure 5.12B).

All patients who suffered relapse or recurrence of infective endocarditis possessed the $PI^{A1/A1}$ genotype, but this was not significant ($PI^{A1/A1}$ 17% vs. $PI^{A1/A2}$ 0%, $PI^{A2/A2}$ 0%, P=0.352). Paradoxically, the $PI^{A1/A2}$ variant was associated with increased development of septic emboli ($PI^{A1/A1}$ 30% vs. $PI^{A1/A2}$ 70% vs. $PI^{A1/A2}$ 0%, P=0.058). There was no association between $PI^{A1/A2}$ genotype and presence of vegetations, vegetation size or mobility, need for surgery, heart failure and mortality when analysed individually (Tables 5.3 and 5.4). Furthermore, the GPIIIa $PI^{A1/A2}$ polymorphism did not influence development of the composite clinical end-point of embolism, heart failure, need for surgery or mortality.

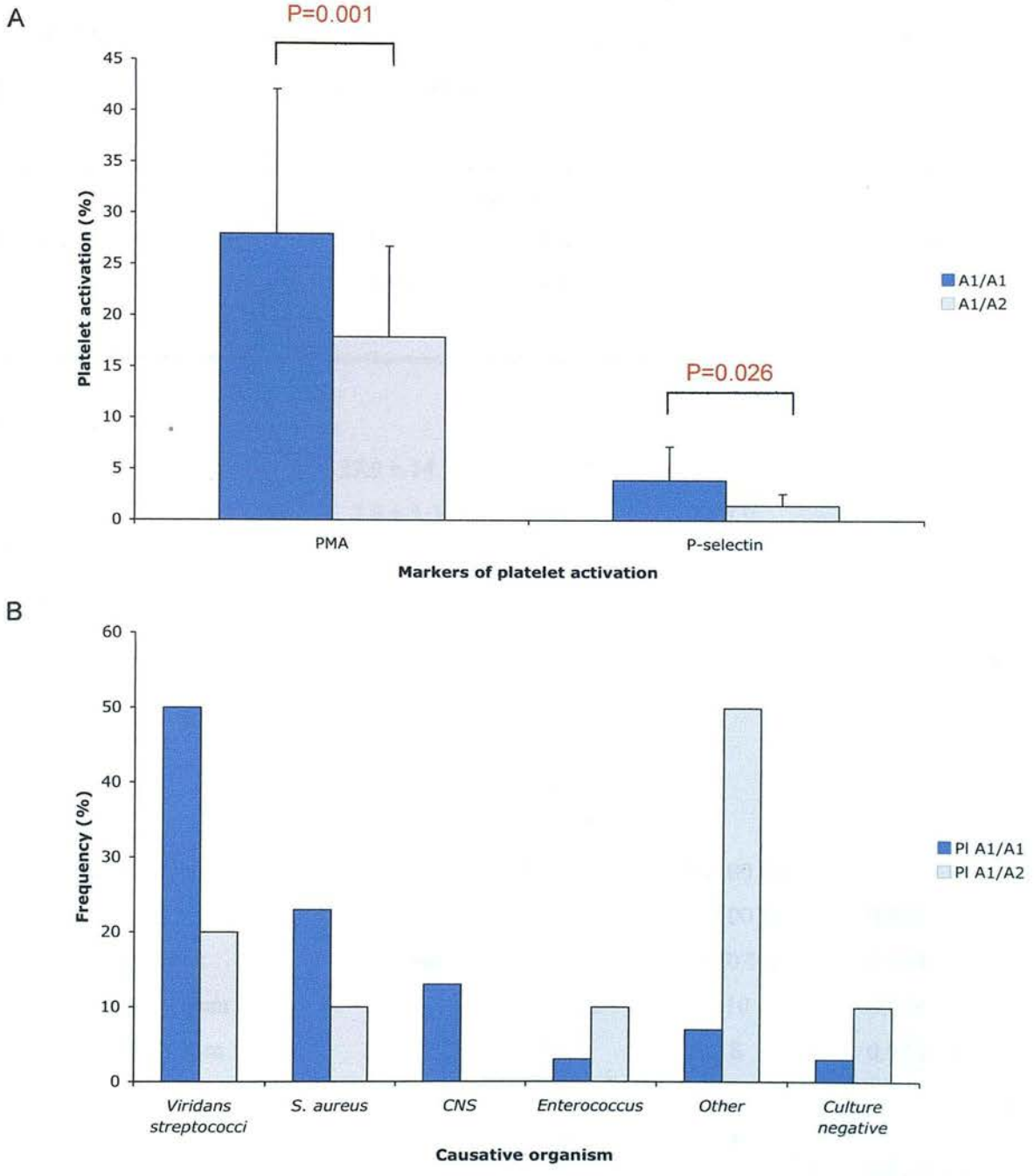


Figure 5.12. Correlation between GPIIIa $PI^{A1/A2}$ genotype and platelet activation (platelet-monocyte aggregate (PMA) formation and platelet P-selectin expression) (A) and causative organism (B) in 40 patients with infective endocarditis. P=0.004 for causative organism. Data for the one $PI^{A2/A2}$ individual are not included. CNS, coagulase-negative staphylococci. See also Table 5.3.

Table 5.3. Correlation between GPIIIa $PI^{A1/A2}$ genotype and clinical characteristics in 41 patients with infective endocarditis for whom genotypic information was available.

Clinical characteristics	GPIIIa $PI^{A1/A2}$ genotype			P value
	A1/A1 n=30	A1/A2 n=10	A2/A2 n=1	
Platelet activation				
PMA, %	27.9 ± 14.1	17.9 ± 8.8	84.0	0.001
P-selectin expression, %	3.9 ± 3.3	1.5 ± 1.1	7.9	0.026
Causative organism				
Streptococci	15 (50 %)	2 (20 %)		
S. aureus	7 (23 %)	1 (10 %)		
Coagulase-negative staphylococci	4 (13 %)	0 (0 %)		0.004
Enterococcus	1 (3 %)	1 (10 %)		
Other	2 (7 %)	5 (50 %)		
Culture negative	1 (3 %)	1 (10 %)	1 (100 %)	
Vegetations	23 (77 %)	8 (80 %)	1 (100 %)	0.845
Multiple vegetations	7 (23 %)	3 (30 %)	0 (0 %)	0.774
Vegetation length, mm *	14 ± 5	20 ± 12	10	0.166
Vegetation width, mm *	9 ± 4	9 ± 3	8	0.946
Vegetation mobility *				
Absent	4	0	1	
Low	11	1		0.110
Moderate	4	1		
Severe	4	4		

* Data on vegetation size and mobility were available for 30 of the 32 subjects (94%) with vegetations and a determined $PI^{A1/A2}$ genotype. PMA, platelet-monocyte aggregates

Table 5.4. Correlation between GPIIIa $PI^{A1/A2}$ genotype and clinical outcome in 41 patients with infective endocarditis for whom genotypic information was available.

Clinical outcome	GPIIIa $PI^{A1/A2}$ genotype			P value
	A1/A1 n=30	A1/A2 n=10	A2/A2 n=1	
Emboli	9 (30 %)	7 (70 %)	0 (0 %)	0.058
Surgery	18 (60 %)	7 (70 %)	0 (0 %)	0.384
Heart failure	10 (33 %)	1 (10 %)	1 (100 %)	0.108
Recurrence or relapse	5 (16 %)	0 (0 %)	0 (0 %)	0.352
Death	6 (20 %)	3 (30 %)	0 (0 %)	0.696
Composite clinical end-point	22 (73 %)	8 (80 %)	0 (0 %)	0.227

Composite clinical end-point, emboli, surgery, heart failure or death

5.3.5.2 The GPIb Kozak sequence polymorphism is not associated with clinical outcome in infective endocarditis

There were no significant correlations between the GPIb Kozak sequence polymorphism and clinical characteristics or outcome in patients with infective endocarditis (Tables 5.5 and 5.6), although there was a trend towards increased heart failure in patients with a C allele (P=0.064, Table 5.6).

5.3.5.3 The HPA-2a/2a genotype is associated with adverse outcome in infective endocarditis

The HPA-2a/2a genotype was associated with increased incidence of the composite clinical end-point of embolism, heart failure, need for surgery and mortality (P=0.020, Table 5.8). Conversely, there was a trend towards increased PMA in patients with the HPA-2a/2b genotype (P=0.056, Table 5.7). There were no associations between GPIb HPA-2 genotype and causative organism, presence of vegetations, vegetation size or vegetation mobility (Tables 5.7 and 5.8).

5.3.5.4 The GPIb VNTR B allele is associated with increased vegetation formation in infective endocarditis

In contrast to results from *in vitro* studies, all patients with VNTR B alleles had vegetations (P=0.006, Table 5.9). Furthermore, there were trends towards increased emboli in patients with the VNTR C/C genotype (P=0.053, Table 5.10). There were no associations between GPIb VNTR genotype and causative organism, vegetation size or mobility or development of the composite clinical end-point (Tables 5.9 and 5.10).

Table 5.5. Correlation between GPIb Kozak genotype and clinical characteristics in 39 patients with infective endocarditis for whom genotypic information was available.

Clinical characteristics	GPIb Kozak genotype			P value
	T/T n=31	T/C n=7	C/C n=1	
Platelet activation				
PMA, %	28.5 ± 17.4	24.2 ± 14.7	24.3	0.826
P-selectin expression, %	3.9 ± 3.4	2.7 ± 3.0	3.0	0.596
Causative organism				
Streptococci	12 (39 %)	3 (43 %)		
S. aureus	7 (23 %)	1 (14 %)	1 (100 %)	
Coagulase-negative staphylococci	3 (10 %)	1 (14 %)		0.830
Enterococcus	2 (6 %)	0 (0 %)		
Other	5 (16 %)	2 (29 %)		
Culture negative	2 (6 %)	0 (0 %)		
Vegetations	23 (74 %)	6 (86 %)	1 (100 %)	0.693
Multiple vegetations	7 (23 %)	1 (14 %)	1 (100 %)	0.162
Vegetation length, mm *	15 ± 8	15 ± 5	15	0.986
Vegetation width, mm *	9 ± 4	11 ± 5	10	0.599
Vegetation mobility *				
Absent	4	1		
Low	9	1	1	0.829
Moderate	3	0		
Severe	7	4		

* Data on vegetation size and mobility were available for 23 of the 30 subjects (77%) with vegetations and a determined GPIb Kozak sequence genotype. PMA, platelet-monocyte aggregates

Table 5.6. Correlation between GPIb Kozak sequence genotype and clinical outcome in 39 patients with infective endocarditis for whom genotypic information was available.

Clinical outcome	GPIb Kozak sequence genotype			P value
	T/T n=31	T/C n=7	C/C n=1	
Emboli	12 (39 %)	3 (43 %)	0 (0 %)	0.711
Surgery	18 (58 %)	5 (71 %)	1 (100 %)	0.585
Heart failure	7 (23 %)	4 (57 %)	1 (100 %)	0.064
Recurrence or relapse	3 (10 %)	1 (14 %)	0 (0 %)	0.883
Death	5 (16 %)	2 (29 %)	1 (100 %)	0.104
Composite clinical end-point	22 (71 %)	5 (71 %)	1 (100 %)	0.817

Composite clinical end-point, emboli, surgery, heart failure or death

Table 5.7. Correlation between GPIb human platelet antigen (HPA)-2 genotype and clinical characteristics in 40 patients with infective endocarditis for whom genotypic information was available.

Clinical characteristics	GPIb HPA-2 genotype 2a/2a n=34	2a/2b n=6	P value
Platelet activation			
PMA, %	25.2 ± 14.3	39.4 ± 23.8	0.056
P-selectin expression, %	3.4 ± 3.2	4.2 ± 4.2	0.983
Causative organism			
Streptococci	15 (44 %)	2 (33 %)	
S. aureus	7 (21 %)	1 (17 %)	
Coagulase-negative staphylococci	3 (8 %)	1 (17 %)	0.858
Enterococcus	2 (6 %)	1 (17 %)	
Other	5 (15 %)	0 (0 %)	
Culture negative	2 (6 %)	1 (17 %)	
Vegetations	26 (76 %)	6 (100 %)	0.184
Multiple vegetations	9 (26 %)	1 (17 %)	0.609
Vegetation length, mm *	16 ± 8	11 ± 3	0.128
Vegetation width, mm *	9 ± 5	7 ± 1	0.336
Vegetation mobility *			
Absent	3	2	
Low	10	1	0.306
Moderate	5	0	
Severe	7	2	

* Data on vegetation size and mobility were available for 30 of the 32 subjects (94%) with vegetations a determined GPIb HPA-2 genotype. PMA, platelet-monocyte aggregates

Table 5.8. Correlation between GPIb human platelet antigen (HPA)-2 genotype and clinical outcome in 40 patients with infective endocarditis for whom genotypic information was available.

Clinical outcome	GPIb HPA-2 genotype		P value
	2a/2a	2a/2b	
	n=34	n=6	
Emboli	15 (44 %)	1 (17 %)	0.206
Surgery	23 (68 %)	2 (33 %)	0.109
Heart failure	9 (26 %)	3 (50 %)	0.246
Recurrence or relapse	4 (12 %)	0 (0 %)	0.376
Death	9 (26 %)	0 (0 %)	0.152
Composite clinical end-point	27 (79 %)	2 (33 %)	

Composite clinical end-point, emboli, surgery, heart failure or death

Table 5.9. Correlation between GPIb variable number of tandem repeat (VNTR) genotype and clinical characteristics in 38 patients with infective endocarditis for whom genotypic information was available.

	GPIb VNTR genotype				
Clinical characteristics	**B/C n=3**	**B/D n=1**	**C/C n=29**	**C/D n=5**	**P value**
Platelet activation					
PMA, %	24.9 ± 10.0	39.9	26.6 ± 17.9	32.4 ± 14.1	0.674
P-selectin expression, %*	3.0 ± 4.1		3.7 ± 3.4	2.9 ± 2.2	0.296
Causative organism					
Streptococci	1 (33 %)	1 (100 %)	10 (35 %)	3 (60 %)	
S. aureus			8 (28 %)		
Coagulase-negative staphylococci	1 (33 %)		1 (3 %)	1 (20 %)	0.499
Enterococcus	1 (33 %)		1 (3 %)		
Other			7 (24 %)		
Culture negative			2 (7 %)	1 (20 %)	
Vegetations	3 (100 %)	1 (100 %)	25 (86 %)	1 (20 %)	0.006
Multiple vegetations	1 (33 %)	0 (0 %)	7 (24 %)	1 (20 %)	0.918
Vegetation length, mm †	9 ± 4	14	16 ± 8	17 ± 4	0.486
Vegetation width, mm †	7 ± 1	6	9 ± 4	11 ± 5	0.463

Clinical characteristics	GPIb VNTR genotype B/C n=3	B/D n=1	C/C n=29	C/D n=5	P value
Vegetation mobility †					
Absent	1		2	0	
Low	1		9	2	0.115
Moderate	0	1	2	2	
Severe	0		7	0	

* P-selectin values were not available for the one B/D patient † Data on vegetation size and mobility were available for 28 of the 30 subjects (93%) with vegetations and a determined GPIb VNTR genotype. PMA, platelet-monocyte aggregates

Table 5.10. Correlation between GPIb variable number of tandem repeat (VNTR) genotype and clinical outcome in 38 patients with infective endocarditis for whom genotypic information was available.

Clinical outcome	GPIb VNTR genotype				P value
	B/C n=3	B/D n=1	C/C n=29	C/D n=5	
Emboli	0 (0 %)	0 (0 %)	15 (52 %)	0 (0 %)	0.053
Surgery	1 (33 %)	1 (100 %)	19 (66 %)	3 (60 %)	0.610
Heart failure	1 (33 %)	1 (100 %)	7 (24 %)	3 (60 %)	0.188
Recurrence or relapse	0 (0 %)	0 (0 %)	3 (10 %)	1 (20 %)	0.814
Death	0 (0 %)	0 (0 %)	6 (21 %)	2 (40 %)	0.542
Composite clinical end-point	1 (33 %)	1 (100 %)	22 (76 %)	4 (80 %)	0.384

Composite clinical end-point, emboli, surgery, heart failure or death

5.3.5.5 The FcγRIIa H131 allele is associated with clinical outcome in infective endocarditis

There were no correlations between H131R genotype and platelet activation, causative organism, presence of vegetations, vegetation characteristics, emboli and the composite clinical end-point (Tables 5.11 and 5.12). However, consistent with the association of the H131 allele with increased bacterium-induced platelet aggregation *in vitro*, presence of this allele was associated with the development of heart failure (P=0.018, Table 5.12). Furthermore, only individuals with an FcγRIIa H131 allele suffered recurrence, relapse or mortality although this was not statistically significant (Table 5.12). The H/R genotype was associated with reduced need for surgery in patients with infective endocarditis (P=0.009, Table 5.12).

Table 5.11. Correlation between FcγRIIa HI31R genotype and clinical characteristics in 41 patients with infective endocarditis for whom genotypic information was available.

Clinical characteristics	FcγRIIa HI31R			P value
	H/H n=11	H/R n=19	R/R n=11	
Platelet activation				
PMA, %	24.1 ± 13.7	29.1 ± 17.2	27.3 ± 18.2	0.656
P-selectin expression, %	3.1 ± 3.3	3.7 ± 2.9	2.9 ± 4.4	0.891
Causative organism				
Streptococci	4 (37 %)	7 (36 %)	5 (45 %)	
S. aureus	2 (18 %)	4 (21 %)	3 (27 %)	
Coagulase-negative staphylococci	1 (9 %)	2 (11 %)	1 (9 %)	0.988
Enterococcus	2 (18 %)	0 (0 %)	0 (0 %)	
Other	1 (9 %)	4 (21 %)	2 (19 %)	
Culture negative	1 (9 %)	2 (11 %)	0 (0 %)	
Vegetations	9 (82 %)	14 (74 %)	9 (82 %)	0.821
Multiple vegetations	3 (27 %)	5 (26 %)	2 (18 %)	0.853
Vegetation length, mm *	21 ± 14	13 ± 5	16 ± 7	0.210
Vegetation width, mm *	8 ± 2	8 ± 4	10 ± 5	0.503
Vegetation mobility *				
Absent	0	5	0	
Low	2	6	4	0.074
Moderate	0	4	0	
Severe	2	2	5	

* Data on vegetation size and mobility were available for 30 of the 32 subjects (94%) with vegetations and a determined FcγRIIa H131R genotype. PMA, platelet-monocyte aggregates

Table 5.12. Correlation between FcγRIIa HI31R genotype and clinical outcome in 41 patients with infective endocarditis for whom genotypic information was available.

Clinical outcome	FcγRIIa HI31R			P value
	H/H n=11	H/R n=19	R/R n=11	
Emboli	2 (18 %)	8 (42 %)	6 (55 %)	0.202
Surgery	10 (91 %)	7 (37 %)	8 (73 %)	0.009
Heart failure	6 (55 %)	6 (32 %)	0 (0 %)	0.018
Recurrence or relapse	1 (9 %)	4 (21 %)	0 (0 %)	0.221
Death	4 (36 %)	5 (26 %)	0 (0 %)	0.098
Composite clinical end-point	10 (91 %)	12 (63 %)	8 (73 %)	0.255

Composite clinical end-point, emboli, surgery, heart failure or death

5.4 Discussion

Host factors such as age, intravenous drug abuse, haemodialysis, valvular heart disease and presence of prosthetic valves increase susceptibility to infective endocarditis (Bashore *et al.*, 2006). However, the influence of host genetic factors on *S. aureus*-induced platelet aggregation and outcome in infective endocarditis are unknown. This study is the first to examine the contribution of platelet receptor polymorphisms to *S. aureus*-platelet interactions, development of and clinical outcome in patients with infective endocarditis.

5.4.1 Effects of the GPIIIa $PI^{A1/A2}$ polymorphism on *S. aureus*-platelet interactions and outcome in infective endocarditis

The GPIIb/IIIa receptor has been implicated in *S. aureus*-platelet adhesion and *S. aureus*-induced platelet aggregation under both low and high shear conditions (Fitzgerald *et al.*, 2006b, George *et al.*, 2006, Pawar *et al.*, 2004). Polymorphisms of this receptor may therefore impact on multiple stages of *S. aureus*-induced platelet aggregation under multiple rheological conditions.

In the current study, the GPIIIa $PI^{A1/A1}$ genotype was associated with increased susceptibility to *S. aureus*-induced platelet aggregation *in vitro*, measured by reduced lag time to platelet aggregation. Importantly, the effect of the PI^{A1} variant on lag time was present in studies using washed platelets, suggesting that the observed effects were independent of differences in plasma protein concentrations (Feng *et al.*, 2001). The effect of the $PI^{A1/A2}$ polymorphism was confined to the final stage of platelet aggregation and did not influence levels of *S. aureus*-platelet binding or activation in healthy volunteers, indicating that this polymorphism modulates cross-linking of fibrinogen to GPIIb/IIIa receptors, similar to its influence on platelet aggregation induced by pharmacological agonists (Feng *et al.*, 1999, Lasne *et al.*, 1997, Michelson *et al.*, 2000).

The $PI^{A1/A2}$ genotype was associated with increased aggregation as compared to the $PI^{A2/A2}$ genotype, suggesting the PI^{A1} allele is dominant. This is in contrast to observations by O'Halloran *et al*, who identified increased transcription of PI^{A2}- rather than PI^{A1}-bearing alleles in heterozygotes, but transcription levels may not correlate with phenotypic effects (O'Halloran *et al.*, 2006).

These observations are consistent with those from published studies, which have identified increased platelet aggregation in response to SFLLRN, ADP, arachidonic acid and the thromboxane A2 analogue U46619 in PI^{A1} homozygotes (Andrioli *et al.*, 2000, Lasne et al., 1997). However, most studies have demonstrated increased platelet aggregation and activation at baseline and in response to ADP and epinephrine in PI^{A2} carriers (Feng *et al.*, 1999, Michelson *et al.*, 2000). These discrepancies may be explained by the use of bacterial agonists and different study populations. There were no significant correlations between GPIIIa $PI^{A1/A2}$ genotype and platelet aggregation induced by ADP or SFLLRN-NH_2 in the current study, possibly due to the use of saturating concentrations of pharmacological agonists (Michelson *et al.*, 2000).

In addition to its association with increased *S. aureus*-induced platelet aggregation *in vitro*, the $PI^{A1/A1}$ genotype was associated with blood culture positivity for staphylococci, and streptococci, the commonest causes of infective endocarditis (Murdoch *et al.*, 2009). These data are consistent with our understanding of the central role of the platelet GPIIb/IIIa receptor in the initial adherence of staphylococci and streptococci to platelets leading to platelet aggregation (see Section 1.4.3.3) (Fitzgerald *et al.*, 2006a, Pampolina & McNicol, 2005). In contrast, the platelet receptors involved in interactions with all of the other organisms, including enterococci, isolated from cases of infective endocarditis in the current study have not yet been identified (Fitzgerald et al., 2006a, Usui et al., 1991a). The GPIIIa $PI^{A1/A1}$ genotype also correlated with increased platelet activation in patients with infective endocarditis but not healthy volunteers, suggesting that platelet activation in infective endocarditis may be directly related to the interaction between pathogenic bacteria and polymorphic platelet receptors *in vivo* under high shear

conditions (Fitzgerald *et al.*, 2006a). The negative correlation between the $PI^{A1/A1}$ genotype and risk of embolic phenomena may also be explained by the increased bacterium-induced platelet aggregation observed with this genotype *in vitro*, which could result in the formation of less mobile and friable vegetations that are less likely to embolise (Di Salvo *et al.*, 2001, Thuny *et al.*, 2005). Admittedly, these correlations may no longer be present on analysis of larger study populations.

Although the GPIIIa $PI^{A1/A2}$ polymorphism correlated with platelet activation and causative organism in patients with infective endocarditis, there was no correlation between genotype and susceptibility to infective endocarditis, consistent with studies that have examined the role of this polymorphism in thrombotic disease and intravascular device infection (Di Castelnuovo *et al.*, 2001, Meisel *et al.*, 2004, Musher *et al.*, 2002). These data indicate that the GPIIIa $PI^{A1/A2}$ polymorphism does not represent a risk factor for the development of infective endocarditis, but may be a predictor of causative organism in affected patients.

5.4.2 Effects of the GPIb Kozak sequence polymorphism on *S. aureus*-platelet interactions and outcome in infective endocarditis

The platelet GPIb receptor is implicated in *S. aureus*-platelet adhesion under high shear (Fitzgerald *et al.*, 2006a, George *et al.*, 2006) and polymorphisms of this receptor may influence the development of *S. aureus*-induced platelet aggregation.

With the use of PRP, the GPIb Kozak sequence T/T genotype was associated with more rapid *S. aureus*-induced platelet aggregation but reduced percentage aggregation on univariate analysis. These findings indicate that the polymorphism may influence different stages of platelet aggregation, particularly as the lag time is considered to be a composite of the time taken for *S. aureus*-platelet adhesion and platelet activation to occur, while percentage aggregation represents the capacity of fibrinogen to cross-link host GPIIb/IIIa platelet receptors. However, GPIb Kozak sequence genotype did not influence *S. aureus*-platelet adhesion or activation,

possibly due to the small study population and the static nature of these assays. Furthermore, the Kozak sequence polymorphism did not influence platelet aggregation on multivariate analysis or when using washed platelets, suggesting that its effects may be mediated via other platelet receptor polymorphisms, for example GPIIIa $PI^{A1/A2}$, or influenced by plasma protein (e.g. vWF) concentration.

These observations are consistent with a study which found the T/T genotype to be associated with increased platelet plug formation *in vitro* (Jilma-Stohlawetz *et al.*, 2003). In the current study, there was no correlation between GPIb Kozak sequence genotype and platelet aggregation induced by ristocetin, a pharmacological agonist which is known to induce platelet aggregation via vWF binding to GPIb, although this association has never previously been determined.

The Kozak sequence polymorphism did not influence the development of infective endocarditis, but there were trends towards increased heart failure in those with a C allele. Previous studies have identified increased risk of ischaemic heart disease, stroke and intravascular device infection in individuals with a C allele (Baker *et al.*, 2001, Kenny *et al.*, 2002, Musher *et al.*, 2002), which may exert its effects via increased platelet surface expression of the GPIb receptor (Afshar-Kharghan *et al.*, 1999).

5.4.3 Effects of the GPIb HPA-2 polymorphism on *S. aureus*-platelet interactions and outcome in infective endocarditis

There were no significant correlations between HPA-2 genotype and *S. aureus*- or ristocetin-induced aggregation of platelets in PRP, consistent with studies which did not identify correlations between HPA-2 genotype and vWF or ristocetin binding or platelet plug formation *in vitro* (Jilma-Stohlawetz et al., 2003, Li et al., 2000a). Given these negative findings, further studies determining *S. aureus*-platelet adhesion, activation and aggregation of washed platelets were not performed.

Prevalence of HPA-2 alleles did not vary between the general healthy population and patients with infective endocarditis, consistent with studies that have failed to demonstrate a correlation between the HPA-2 polymorphism and the development of thromboembolic disease (Meisel *et al.*, 2004, Ozelo *et al.*, 2004b). However, the HPA-2a/2a genotype was associated with increased incidence of the composite clinical end-point of embolism, heart failure, need for surgery and mortality, indicating that this genotype may represent a risk factor for adverse outcome in infective endocarditis. However, the results of this study need to be interpreted with caution due to the limited study population size.

5.4.4 Effects of the GPIb VNTR polymorphism on *S. aureus*-platelet interactions and outcome in infective endocarditis

There was increased *S. aureus*- and ristocetin-induced aggregation of platelets heterozygous for a D allele, consistent with previous studies that identified associations of the C/D genotype with platelet plug formation (Jilma-Stohlawetz *et al.*, 2003) and the development of MI or intravascular device infection (Musher *et al.*, 2002, Ozelo *et al.*, 2004b). A possible mechanism for the pro-aggregatory effects of the D allele includes closer proximity of the GPIb receptor to the platelet surface, facilitating platelet aggregation. Given the small number of individuals with D alleles in the current study, the effects of GPIb VNTR genotype on aggregation of washed platelets, *S. aureus*-platelet adhesion or activation *in vitro* were not characterised. A larger study population will be required to ascertain whether the VNTR D allele influences *S. aureus*-platelet interactions, particularly under high shear conditions.

Prevalence of VNTR alleles did not predict the development of infective endocarditis, consistent with observations from studies examining vascular disease (Baker *et al.*, 2001, Cadroy *et al.*, 2001, Carter *et al.*, 1998, Kenny *et al.*, 2002). In contrast to aggregation studies, presence of a B allele was significantly associated with vegetation development in patients with infective endocarditis, while all

patients who developed embolic complications had the C/C genotype. The pro-aggregatory role of B or C alleles has been identified in other studies (Gonzalez-Conejero *et al.*, 1998, Mikkelsson *et al.*, 2001).

5.4.5 Effects of the FcγRIIa H131R polymorphism on *S. aureus*-platelet interactions and outcome in infective endocarditis

The FcγRIIa receptor plays a central role in *S. aureus*-induced platelet activation (Fitzgerald *et al.*, 2006a). An association of the FcγRIIa H131 allele with increased *S. aureus*-induced platelet aggregation was identified in the current study. However, these findings were not reproduced in studies using washed platelets, suggesting that the effects of the H131R polymorphism may be influenced by plasma components, such as IgG, which cross-links with the FcγRIIa receptor to induce platelet activation (Fitzgerald *et al.*, 2006a). Alternatively, the small study population may have been a limiting factor in washed platelet studies.

The data generated with PRP in the current study are consistent with other platelet studies which have demonstrated increased binding of the H131 FcγRIIa platelet receptor to human IgG_2 and IgG_3, accounting for the increased *S. aureus*-platelet interactions observed with this allele in the current study (Chen *et al.*, 2003, van der Pol & van de Winkel, 1998). However, Chen *et al* did not identify any increase in platelet aggregation with the H131 allele (Chen *et al.*, 2003). A previous study by McNicol *et al* failed to identify a correlation between FcγRIIa H131R genotype and ability of *S. sanguinis* strains to induce platelet aggregation *in vitro*, but they were limited by a small study population of 14 donors (McNicol *et al.*, 2006).

Baseline platelet activation in the current study did not vary with FcγRIIa H131R genotype, similar to observations by Chen *et al* (Chen *et al.*, 2003). Consistent with observations from *in vitro* studies, all patients who suffered heart failure, relapse, recurrence or mortality carried an H131 allele, but this was not statistically significant.

Prevalence of H131R alleles in patients with infective endocarditis was similar to healthy volunteers, consistent with studies analysing invasive pneumococcal or meningococcal disease and coronary artery disease (Brouwer *et al.*, 2009, Karakas *et al.*, 2009). The role of the FcγRIIa receptor in sepsis is complex as it is also expressed on the surface of leucocytes and plays a central role in the recognition of opsonising antibody leading to phagocytosis (Bredius *et al.*, 1993, van der Pol & van de Winkel, 1998). Furthermore, the H131 allele has been associated with increased phagocytosis of encapsulated organisms and *S. aureus* (Bredius *et al.*, 1993, Sanders *et al.*, 1995). Thus, the apparent pro-aggregatory role of the FcγRIIa H131 allele identified *in vitro*, may be counteracted to an extent by its importance in innate immune defence, which could account for the protective effect of the homozygote state on need for surgery in this study.

5.4.6 Multivariate analysis of the role of platelet receptor polymorphisms in *S. aureus*-platelet interactions

Although most of the polymorphic platelet receptors had an influence on *S. aureus*-induced platelet aggregation in the current study, statistical significance may have been exaggerated by the multiple comparisons performed on univariate analysis. It is feasible that some of these correlations were identified as a chance finding amongst multiple assessed variables. In order to address these issues, multivariate analysis of the results obtained from PRP aggregation studies was conducted. The same could not be done for results obtained from washed platelet, flow cytometric or patient studies due to small population sizes.

On multivariate analysis, the GPIIIa PI^{A1}, VNTR D and FcγRIIa H131 alleles were significantly associated with increased platelet aggregation. In addition, there was an interactive effect of the GPIIIa $PI^{A1/A2}$ and GPIb Kozak sequence polymorphisms on *S. aureus*-induced platelet aggregation. Specifically, time to aggregation was reduced in platelets with the $PI^{A1/A1}$ genotype, $PI^{A1/A2}$ and Kozak sequence T/T genotype or $PI^{A2/A2}$ platelets carrying the Kozak sequence T/C genotype.

The possible mechanisms for this interaction can be examined by understanding platelet receptor interactions at the molecular level. vWF binding to GPIb and clustering of the GPIb receptor increase GPIIb/IIIa ligand-binding affinity via increased 'inside-out' signalling, mediated by calcium oscillations and phosphorylation of kinases (Arya *et al.*, 2003, Kasirer-Friede *et al.*, 2004, Kasirer-Friede *et al.*, 2002). Furthermore, the ability of GPIb to bind vWF results in increased availability of the GPIIb/IIIa receptor to bind fibrinogen alone, potentiating thrombus formation (Wu *et al.*, 2000). It therefore appears that there is a synergistic effect between these two platelet integrin receptors, but the mechanisms by which they are influenced by platelet receptor polymorphisms are unknown. It can be speculated that increased platelet plug formation with the Kozak sequence T/T genotype may enhance activation of GPIIIa $Pl^{A1/A2}$ platelets, while increased surface expression of the GPIb receptor conferred by the Kozak sequence C allele may facilitate ligand-binding to $Pl^{A2/A2}$ platelets.

Additive effects of polymorphisms have been observed in other studies. GPIIIa $Pl^{A1/A2}$ and GPIa/IIa receptor polymorphisms together represent risk factors for the development of arterial thrombosis in patients with primary or secondary anti-phospholipid syndrome (Jimenez *et al.*, 2008). In addition, Saidi *et al*, identified correlations between the presence of variant alleles in GPIIIa $Pl^{A1/A2}$, GPIb HPA-2 and GPIIb/IIIa HPA-3 and the development of ischaemic stroke in a large study (Saidi *et al.*, 2008). Iniesta *et al* determined that combinations of polymorphisms of GPIIIa $Pl^{A1/A2}$ and GPIb HPA-2 influenced the development of subarachnoid haemorrhage (Iniesta *et al.*, 2004). However, this is the first time that an interactive effect between GPIIIa $Pl^{A1/A2}$ and GPIb Kozak sequence polymorphisms has been observed. It will be important to determine whether these interactive effects are present with larger study populations of healthy volunteers for *in vitro* studies, as well as clinical outcome in patients with infective endocarditis.

5.4.7 Conclusions

In summary, the GPIIIa PI^{A1}, VNTR and FcγRIIa H131 alleles are associated with enhanced *S. aureus*-platelet interactions and adverse outcome in infective endocarditis. Furthermore, the HPA-2a/2a genotype was associated with increased development of the composite clinical end-point of embolism, heart failure, need for surgery and mortality in infective endocarditis

However, there were limitations to the current study. Some of the observations from PRP aggregation studies were not reproduced in washed platelet studies, possibly due to the small study population in the latter cohort. Population size also limited the ability to conduct multivariate analysis on acquired data. The contrasting findings regarding the role of GPIb polymorphisms *in vitro* and *in vivo* in the current study highlight the drawbacks of using a low shear system for functional platelet assays, particularly as the GPIb receptor has been implicated in *S. aureus*-platelet interactions under high shear conditions (George *et al.*, 2006, Pawar *et al.*, 2004). High shear assays, such as those outlined in Section 3.4.5 may help to further characterise the influence of platelet receptor polymorphisms on *S. aureus*-induced platelet aggregation.

Nevertheless, this study has identified that platelet receptor polymorphisms may be a determinant of *S. aureus*-platelet interactions *in vitro* and outcome in infective endocarditis. These observations merit further investigation with large numbers of patients with infective endocarditis, to determine the role of platelet receptor polymorphisms as prognostic markers in bacteraemia and infective endocarditis. Given these observations, anti-platelets targeting the GPIIb/IIIa, GPIb and FcγRIIa receptors should be explored as adjuncts to antibiotic therapies in infective endocarditis.

CHAPTER 6

GENERAL DISCUSSION

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6.1 Introduction

This aim of this research was to determine the influence of selected host-pathogen interactions on susceptibility to infective endocarditis. Specifically, the objectives were to analyse the effect of *S. aureus* growth in blood on platelet aggregation *in vitro*, the correlation between platelet activation levels *in vivo* and the development of infective endocarditis and the influence of platelet receptor polymorphisms on *S. aureus*-induced platelet aggregation and outcome in infective endocarditis. Figure 6.1 depicts the host and bacterial factors investigated in the current research and their impact on the various stages of *S. aureus*-platelet interactions contributing to the pathogenesis of infective endocarditis. The implications of these findings are discussed below in further detail.

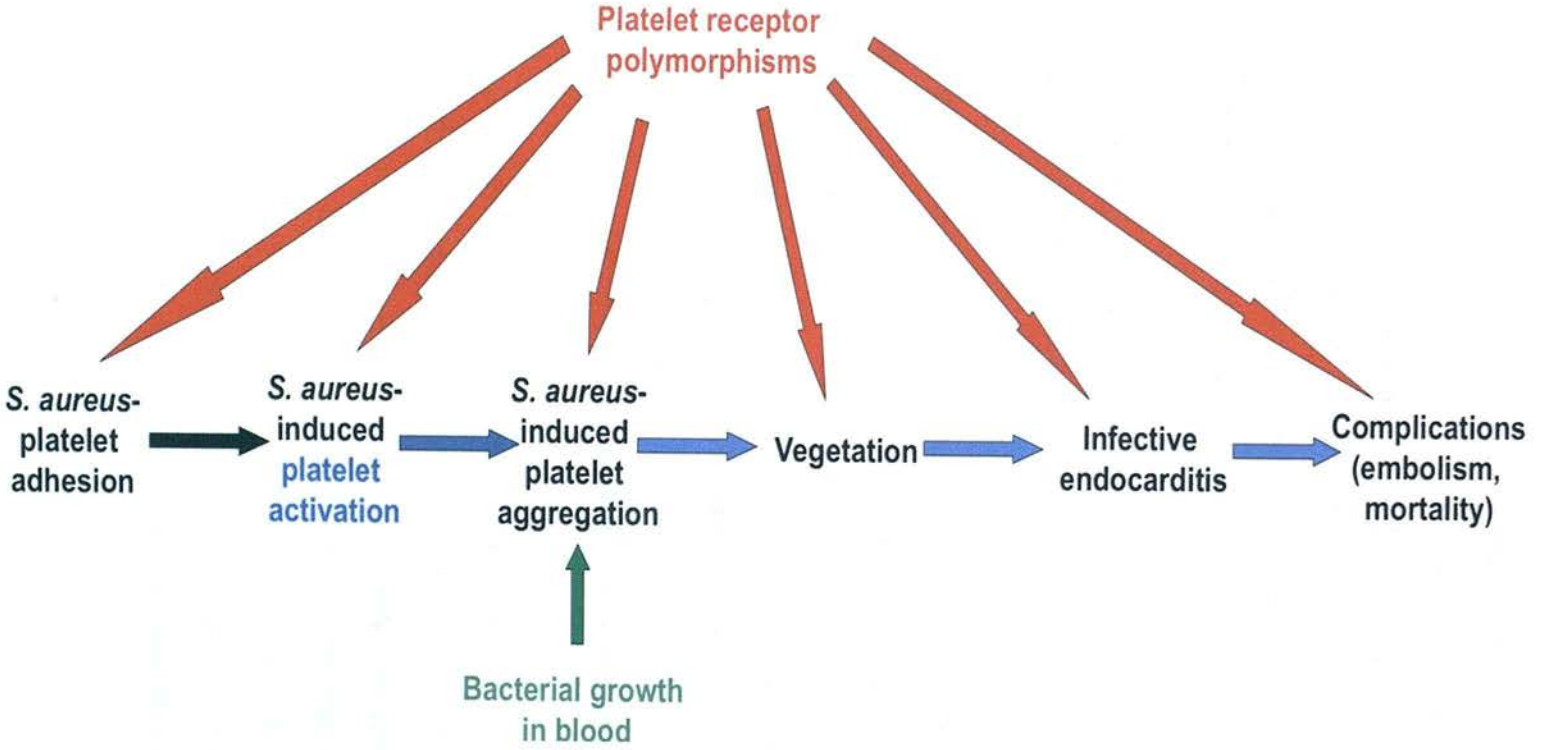

Figure 6.1. Current understanding of the *S. aureus*-platelet interactions leading to vegetation formation in infective endocarditis (black) and contributory host and pathogen factors investigated in the current study. Factors investigated include the effect of bacterial growth in blood on platelet aggregation (green), the correlation between host platelet activation levels *in vivo* and development of infective endocarditis (blue) and the influence of host platelet receptor polymorphisms on *S. aureus*-platelet interactions and outcome in infective endocarditis (red).

6.2 Host-pathogen interactions influence susceptibility to and outcome in infective endocarditis

This research demonstrates that *S. aureus*-induced platelet aggregation does not predict the development of infective endocarditis, that platelet receptor genotype is associated with clinical outcome in infective endocarditis and that *in vivo* platelet activation inversely correlates with the development of infective endocarditis and associated complications. Collectively, these data suggest that the pathogenesis and outcome of infective endocarditis may be more dependent on host factors than the bacterial factors investigated in the current study.

The fact that *S. aureus*-induced platelet aggregation did not correlate with the capacity of strains to cause infective endocarditis in humans, suggests that the development of *S. aureus* infective endocarditis may instead have a greater dependence on bacterial platelet microbicidal protein (PMP)-resistance, which has been demonstrated to correlate with the development of staphylococcal and streptococcal infective endocarditis (Wu *et al.*, 1994). Alternatively, host factors such as variation in innate immunity, plasma protein concentration, platelet-specific factors and underlying endothelial damage may have a more profound influence on the outcome of bacterium-platelet interactions (Brouwer *et al.*, 2009, Feng *et al.*, 2001, Meisel *et al.*, 2004, van der Pol & van de Winkel, 1998).

Consistent with this theory, host factors examined in the current study correlated with adverse outcome in infective endocarditis. Specifically, platelet receptor genotype was associated with *S. aureus*-induced platelet aggregation *in vitro* and the development of complications in patients with infective endocarditis. However, platelet receptor polymorphisms did not predict the development of infective endocarditis, suggesting that polymorphic platelet receptors solely correlate with outcome in infective endocarditis, while other host factors influence susceptibility to infective endocarditis.

In this study, reduced *in vivo* platelet activation, likely representing the outcome of bacterium-platelet interactions in the host, correlated with the development of infective endocarditis. In contrast, platelet activation was increased in patients requiring valve replacement for infective endocarditis. These discrepant findings are most likely due to the variable timing of patient recruitment and the small study population sizes. However, these observations are also consistent with the dual role of platelets, which are known to interact with bacteria leading to vegetation formation in infective endocarditis, while also contributing to host innate immunity (Fitzgerald *et al.*, 2006a, Yeaman, 1997). It can be speculated that the presence of reduced platelet activation in patients with infective endocarditis may lead to decreased PMP release, reducing innate immunity and increasing the risk of developing infective endocarditis. Conversely, the association of increased platelet activation and polymorphic platelet receptors with bacterium-induced platelet aggregation and clinical outcome in patients with infective endocarditis, suggests that platelets may predominantly contribute to thrombosis in the later stages of the disease process.

Temporal adaptation of the role of platelets in infective endocarditis may be due to fluctuation in the dynamics of host-pathogen interactions, such as variation in the bacterium:platelet ratio during the course of disease (Trier *et al.*, 2008). Alternatively, the institution of antimicrobial therapy, which enhances host immunity and in some cases alters platelet count and function, may account for variable platelet function in infective endocarditis (Rohmann *et al.*, 1997, Von Drygalski *et al.*, 2007). Either way, it appears that platelets play a complex role in the pathogenesis of infective endocarditis, which may account for the failure of aspirin to consistently reduce embolism and mortality in clinical trials of infective endocarditis (Chan *et al.*, 2003, Chan *et al.*, 2008, Eisen *et al.*, 2009, Pepin *et al.*, 2009). It is feasible that the benefits of anti-platelet therapy in infective endocarditis may depend upon the presence of underlying host risk factors or the stage of the disease process.

6.3 Novel therapeutic approaches targeting host-pathogen interactions in infective endocarditis

In addition to conventional treatment modalities, such as antibiotics, valve replacement or repair (Habib *et al.*, 2009), these results highlight the potential benefit of additional therapeutics in infective endocarditis. Such treatments should focus on targeting different stages and factors predisposing to infective endocarditis, with specific aims to prevent the development of infective endocarditis and reduce complication rates.

Minimisation of complication rates in infective endocarditis may result from therapeutics that inhibit bacterium-platelet interactions at later stages of the disease process. Although the potential benefits of aspirin therapy in infective endocarditis have been evaluated, the results from clinical trials have been disappointing and inconclusive, largely due to small study populations and the retrospective nature of such studies (see Section 1.7.2). Future studies should examine the clinical efficacy of both short- and long-term aspirin therapy in large, multi-centre, randomised controlled trials.

A number of *in vitro* studies have demonstrated that antibodies against GPIIb/IIIa, GPIb and FcγRIIa platelet receptors inhibit staphylococcal- and streptococcal-induced platelet activation and aggregation (Fitzgerald et al., 2006b, Kerrigan et al., 2002, O'Brien et al., 2002a, Sjobring et al., 2002), but it is not known whether they have a role in the treatment of infective endocarditis in animal models or in humans. However, GPIIb/IIIa antagonists are extensively used in the treatment of thrombosis in acute coronary syndromes, myocardial infarction and post-percutaneous coronary intervention (Tricoci & Peterson, 2006), while anti-GPIb monoclonal antibodies significantly reduced infarct size and improved functional outcome in an experimental animal model of cerebrovascular disease (Kleinschnitz *et al.*, 2007). It is feasible that anti-platelets targeting the GPIIb/IIIa, GPIb and FcγRIIa receptors may also reduce the severity of infective endocarditis and their therapeutic potential should be explored in multi-centre clinical trials. However, the efficacy of anti-

platelet therapy in infective endocarditis may depend upon underlying host platelet receptor genotype.

Pharmacogenetics, or the influence of genetic variation on response to drug therapy, is a recently identified phenomenon, illustrated by the influence of platelet receptor genotype on anti-platelet efficacy (Topol, 2008). GPIIIa Pl^{A1} carriers are more sensitive to the effects of GPIIb/IIIa receptor antagonists, clopidogrel and aspirin than GPIIIa $Pl^{A2/A2}$ individuals (Feher *et al.*, 2009, Motovska *et al.*, 2009), and as increased *S. aureus*-induced platelet activation and aggregation has been identified in $Pl^{A1/A1}$ platelets, it can be speculated that use of anti-platelets in patients carrying this genotype may improve outcome in infective endocarditis. In addition, aspirin sensitivity is increased in GPIb VNTR C/C patients with cerebrovascular disease (Jin *et al.*, 2009). Considering that patients with infective endocarditis carrying VNTR C alleles were more likely to develop emboli in the current study, these individuals may derive greater benefit from aspirin therapy. Furthermore, three human monoclonal antibodies against FcγRIIa bind more avidly to the H131 form of FcγRIIa than the R131 form, and may prevent platelet activation, aggregation and minimise adverse outcome in patients with infective endocarditis (Bachelot *et al.*, 1995). Pharmacogenetics may account for the variable effects of aspirin on clinical outcome in patients with infective endocarditis (Chan *et al.*, 2003, Chan *et al.*, 2008, Eisen *et al.*, 2009, Pepin *et al.*, 2009), and it is feasible that determination of platelet receptor genotype in patients with infective endocarditis could help identify those that would derive the greatest benefit from tailored anti-platelet therapy.

The prevention of infective endocarditis and minimisation of related complications may also involve enhancement of innate immunity with immunomodulatory drugs or vaccines (Ballow & Nelson, 1997). Although the ability of *S. aureus* strains to induce platelet aggregation *in vitro* did not correlate with disease causation in the current study, the ability of bacteria to interact with platelets is likely to contribute to vegetation formation, which may ultimately influence clinical outcome in infective endocarditis. The capacity of FnBPs to mediate platelet aggregation following *S. aureus* growth in blood suggests that they may represent therapeutic targets in

infective endocarditis. Current vaccines against *S. aureus* capsule and ClfA have been unsuccessful in clinical trials of *S. aureus* infection (Schaffer & Lee, 2008). Anti-FnBP antibodies reduce bacterial vegetation densities in rat models of infective endocarditis (Rennermalm *et al.*, 2001, Schennings *et al.*, 1993), and vaccination of mice with the A domains of FnBPA and ClfA reduces aortic patch infection (Arrecubieta *et al.*, 2008), but their ability to prevent disease in humans is unknown. Unfortunately, development of functional monoclonal antibodies against FnBPs has proven to be a challenge, partly due to sequence diversity of the A domain, resulting in antigenic variation (Loughman *et al.*, 2008). Furthermore, although the fibronectin-binding D domains are largely conserved in FnBPs, they have low immunogenicity when unbound, as an immunogenic neoepitope is only formed after MSCRAMM adherence to fibronectin, resulting in the formation of the ligand-induced binding site that is recognised by antibodies (Casolini *et al.*, 1998, Flock, 1999, Sinha & Herrmann, 2005). The dependence on a ligand-induced binding site for monoclonal antibody binding to FnBPs may limit vaccine efficacy.

The benefits of antibiotic prophylaxis in infective endocarditis have recently been questioned in national and international guidelines, primarily due to the lack of convincing evidence to date (Habib *et al.*, 2009, National Institute for Health and Clinical Excellence, 2008). Cardiologists and dentists have attempted to implement these new guidelines, often with inconsistency and judicious use on a case-by-case basis, resulting in confusion for patients and clinicians alike. Given the results from the current study, and the ability of antimicrobials to interfere with bacterial gene transcription and platelet function (see Section 1.7.1) (Smith *et al.*), it can be speculated that in addition to their bacteriocidal and bacteriostatic effects, prophylactic antibiotics may also interfere with bacterium-platelet interactions that culminate in vegetation formation. The potential benefits of prophylactic antimicrobials may also vary with underlying host susceptibility such as the presence of valvular heart disease or platelet receptor polymorphisms. Following the change in local and international guidelines on antibiotic prophylaxis for infective endocarditis, several large national observational studies have been organised to

determine whether this influences the course of infective endocarditis (Habib *et al.*, 2009).

6.4 Future perspectives

Future studies should aim to expand on findings from this research and investigate other host and pathogen factors that potentially influence susceptibility to infective endocarditis (Figure 6.2). For example, platelet receptor polymorphisms may influence *Staphylococcus*- and *Streptococcus*-platelet interactions under high shear. Furthermore, host characteristics such as polymorphisms of immune system components (e.g. toll-like receptors, complement factors and cytokines), variation in coagulation and fibrinolytic cascade components (e.g. polymorphisms of plasminogen activator, factor V and protein C), platelet receptor density, plasma protein concentration, PMP and IgG titres (Figure 6.2A-E) may modulate the outcome of bacterium-platelet interactions in infective endocarditis (Brouwer *et al.*, 2009, Dankert *et al.*, 2001, Feng *et al.*, 2001, Foster, 2005, O'Halloran *et al.*, 2006). If these factors are found to influence the development of infective endocarditis, this could result in the advent of treatment modalities such as synthetic cytokine or complement analogues, monoclonal antibodies, and the use of anti-thrombotic or fibrinolytic agents in the management of infective endocarditis (Ballow & Nelson, 1997, Kleinschnitz *et al.*, 2007, Tricoci & Peterson, 2006).

It is important to remember that like platelets, host antibodies may either contribute to or inhibit the pathogenesis of infective endocarditis. Although binding of host antibodies against MSCRAMMs to the platelet FcγRIIa receptor facilitates platelet aggregation (see Figure 1.7), higher antibody titres may facilitate bacterial opsonisation in addition to blocking MSCRAMM ligand-binding sites, preventing platelet activation (Fitzgerald *et al.*, 2006a, Ford *et al.*, 1997, Sjobring *et al.*, 2002, Sullam *et al.*, 1988). Furthermore, IgG levels may vary with FcγRIIa H131R platelet receptor genotype, particularly as the H131 allele has been demonstrated to bind human IgG_2 and IgG_3 with greater affinity (van der Pol & van de Winkel, 1998). It is

of paramount importance to determine whether host IgG titres influence *S. aureus*-induced platelet activation, aggregation and the pathogenesis of infective endocarditis, as this may influence the therapeutic potential of vaccines against host and bacterial components in infective endocarditis.

Future research should also investigate the bacterial factors influencing pathogen survival and vegetation formation following bacterial growth in human blood. For example, the effect of growth medium on *clfA* gene transcription observed in this study may extend to other bacterial genes, including those encoding components mediating platelet and (sub)-endothelium interactions, tissue invasion and destruction, coagulation, nutrient acquisition and evasion of host innate immunity (Figure 6.2F-J). If specific bacterial factors are found to be essential for bacterial survival or the pathogenesis of infective endocarditis, novel therapeutics involving passive or active immunisation against those components may dramatically improve the outcome of bacteraemia and infective endocarditis.

It is clearly a priority to elucidate the dynamic interactive effects of host and bacterial components that facilitate bacterial survival in the human host, microbial colonisation and invasion of host valvular tissue, and vegetation formation (Figure 6.2). For example, the outcome of interactions between host innate immunity and bacterium evasion of innate defences, exemplified by the interplay of host platelet PMP release and bacterium PMP-resistance (Figure 6.2E-F), may determine the outcome of bacteraemia. However, such experiments should be performed under conditions mimicking those present *in vivo*, which can be achieved by analysing bacterium-platelet-(sub)-endothelium interactions using bacteria grown in blood, either in high shear flow chambers or by use of animal models of infective endocarditis.

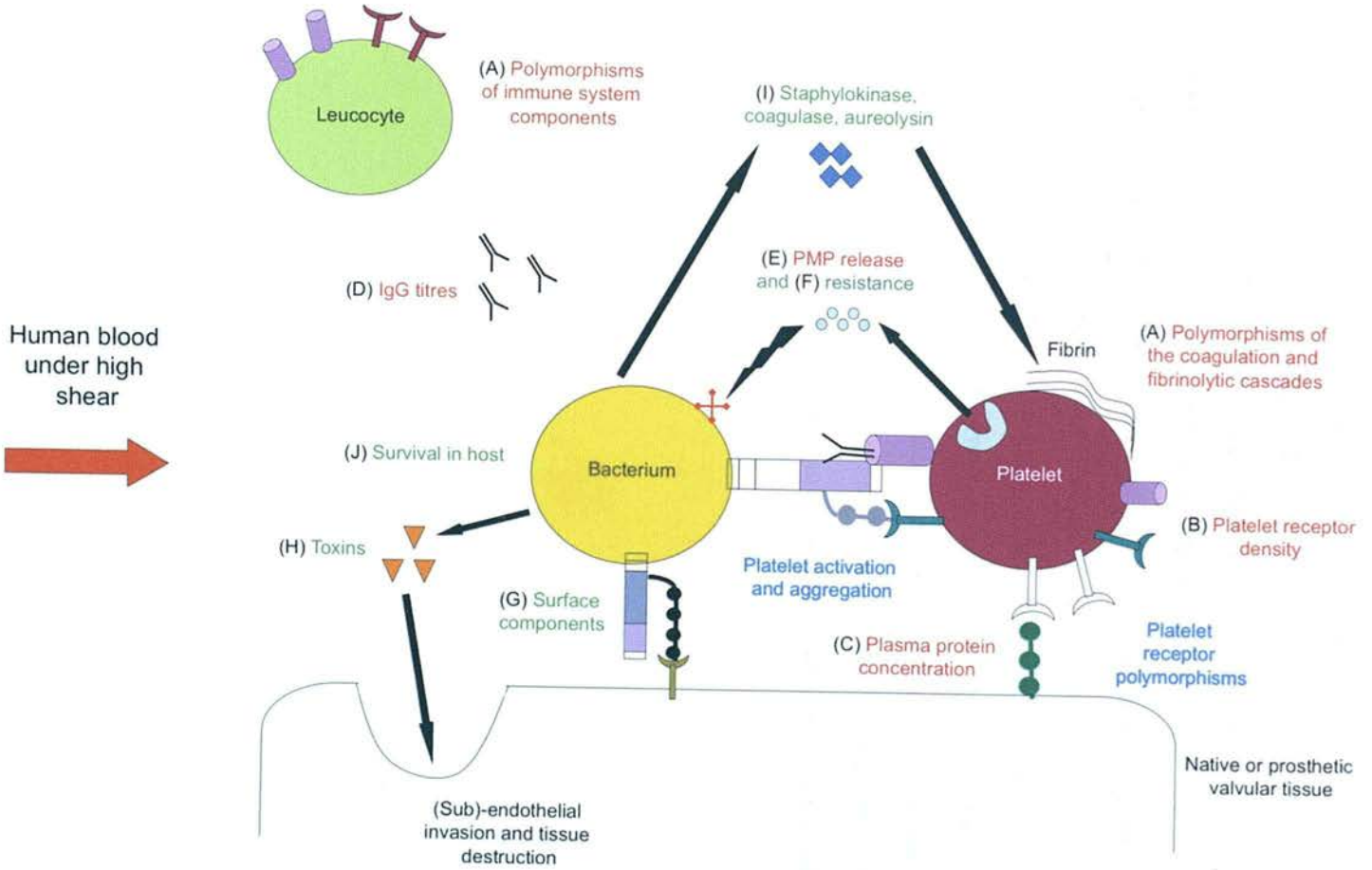

Figure 6.2. Host and pathogen factors that potentially influence bacterium-platelet-(sub)endothelium interactions in infective endocarditis. Factors investigated in the current study are delineated in blue text, while other host (red text, A-E) and pathogen (green text, F-J) factors that merit further investigation in blood under high shear conditions are also outlined.

Rat or rabbit models are often seen as 'gold standard' models of infective endocarditis, but do not accurately reflect the human *in vivo* state. For example, animal models routinely involve catheterisation to facilitate artificial induction of valvular endothelial damage, which enables formation of a sterile thrombus that is subsequently infected by bacteria at a high concentration (Piroth *et al.*, 2008, Siboo *et al.*, 2005). However, the precise dynamics and timing of bacterium-platelet-sub-(endothelium) interactions contributing to vegetation formation in infective endocarditis are still not fully understood (see Section 1.4.3). The structure of cardiac valves also varies amongst different mammals and in comparison to the human heart, the rabbit heart has a thinner and shorter intervalvular septum (Walmsley, 1978). Furthermore, not all animals spontaneously acquire valvular heart disease that would otherwise predispose to infective endocarditis in humans (Donnelly, 2008). Human platelets also take longer to aggregate in response to *S. sanguinis* than rat or rabbit platelets (Manning *et al.*, 1994), and have been reported to release different PMPs as compared to rabbit platelets (Tang *et al.*, 2002).

6.5 Conclusion

S. aureus infective endocarditis is a life-threatening condition and an urgent public health issue. The identification of contributing host and bacterial factors is crucial to enable the development of novel therapeutics against infective endocarditis. Taken together, the results of this research suggest that the host factors investigated in the current study may be of greater importance than the investigated bacterial factors in determining susceptibility to and prognosis in infective endocarditis, as host platelet activation and platelet receptor genotype correlated with disease development and severity in infective endocarditis. Furthermore, it appears that platelets play a dual role in infective endocarditis, which may limit the efficacy of anti-platelet therapy.

This research has highlighted the necessity of a unified understanding of the interaction of host and bacterial components in the development of infective endocarditis. Such integrated studies will enhance our knowledge of the factors influencing the pathogenesis of infective endocarditis, with major implications for patient screening and the development of novel prophylactics and therapeutics against this fatal condition.

APPENDIX 1

S. AUREUS VIRULENCE FACTORS

Table S1.1. Secreted and surface virulence factors expressed by *S. aureus*. Table adapted from (Clarke & Foster, 2006, Dinges *et al.*, 2000, Lowy, 1998, Novick, 2003, Foster, 2005) with additional references as highlighted.

Virulence factor	Secreted/ surface	Function	References
Toxins			
α-toxin	Secreted	Lyses erythrocytes and platelets via pore formation Mediates neurotoxicity of rabbit nerves Reduces myocardial contractility and perfusion Induces platelet aggregation Associated with reduced virulence in an animal model of infective endocarditis Implicated in ARDS, keratitis, mastitis, peritonitis and murine lung infection	(Bayer *et al.*, 1997, Bhakdi *et al.*, 1988, Dinges *et al.*, 2000, Grandel *et al.*, 2009)
β-toxin	Secreted	Lyses sheep erythrocytes via sphingomyelinase activity Associated with increased bacterial growth in the murine mammary gland	(Dinges *et al.*, 2000)
δ-toxin	Secreted	Induces haemolysis and dermonecrosis Role in clinical disease unknown	(Dinges *et al.*, 2000)

Virulence factor	Secreted/ surface	Function	References
γ-toxin	Secreted	Lyses erythrocytes and leucocytes Role in clinical disease unknown	(Dinges *et al.*, 2000, Foster, 2005)
Superantigens	Secreted	Include TSST-1 and enterotoxins (SEA to SER, SEU) Bind MHC II complexes on antigen-presenting cells without the requirement for antigen Implicated in TSS, staphylococcal food poisoning, SIDS, Kawasaki's disease and arthritis	(Dinges *et al.*, 2000, Fraser & Proft, 2008)
Exfoliative toxins	Secreted	Include epidermolytic toxins A and B Implicated in dermatological conditions such as blisters, exfoliation, pemphigus and staphylococcal scalded skin syndrome	(Dinges *et al.*, 2000)
Panton-Valentine leucocidin (PVL)	Secreted	Toxic to leucocytes Implicated in severe cutaneous infection	(Foster, 2005)
Other leucocidins	Secreted	Include leucocidins E-D and M/F-PV-like Roles in clinical disease unknown	(Dinges *et al.*, 2000, Foster, 2005)

Virulence factor	Secreted/ surface	Function	References
Enzymes			
Proteases	Secreted	Include serine protease-like protease, V8 protease, cysteine protease and staphopain Cleave proteins	(Lowy, 1998, Novick, 2003)
Aureolysin (metalloprotease)	Secreted	Cleaves and inactivates human defensin peptide and clumping factor B (ClfB) Role in clinical disease unknown	(Foster, 2005, Ni Eidhin *et al.*, 1998)
Lipases	Secreted	Include phospholipase C Cleave lipids	(Lowy, 1998, Novick, 2003)
Hydrolases	Secreted	Include glycerol ester hydrolase Cleave water molecules	(Clarke & Foster, 2006)
Hyaluronidase	Secreted	Cleaves hyaluronic acid, facilitates *S. aureus* dissemination within the host	(Lowy, 1998, Novick, 2003)
Nucleases	Secreted	Include thermonuclease Cleave nucleic acids for nutrition	(Dinges *et al.*, 2000, Novick, 2003)

Virulence factor	Secreted/ surface	Function	References
Collagenase	Secreted	Cleaves collagen	(Dinges *et al.*, 2000)
Coagulase	Secreted	Prothrombin activator, converts fibrinogen to fibrin No role in the pathogenesis of IE	(Lowy, 1998, Moreillon *et al.*, 1995)
β-lactamase	Secreted	Inactivates β-lactam antibiotics such as penicillin	(Lowy, 2003)
Staphylokinase	Secreted	Encoded on phage Plasminogen activator, resulting in clot dissolution. Role as a thrombolytic drug explored. Inactivates complement and IgG Role in clinical disease unknown	(Bokarewa *et al.*, 2006, Foster, 2005)
Other secreted proteins			
CHIPS	Secreted	Binds complement Prevents neutrophil chemotaxis	(Foster, 2005)
SCIN	Secreted	Inactivates complement	(Foster, 2005)

Virulence factor	Secreted/ surface	Function	References
SSLs	Secreted	Sequence similarity to TSST-1 Encoding genes predominantly on pathogenicity islands Bind IgA, complement components Inhibit neutrophil binding to endothelium via PSGL-1 SSL-5 induces platelet aggregation	(de Haas *et al.*, 2009, Fraser & Proft, 2008)
Extracellular adherence protein (Eap)	Secreted	Binds ICAM-1 on endothelial cells, preventing leucocyte recruitment and transmigration Binds fibrinogen, fibronectin, vitronectin, collagen, and thrombospondin Anti-angiogenic Binds T cells, reducing proliferation and inducing apoptosis of T and B cells Interferes with healing of deep wounds	(Foster, 2005, Sinha & Herrmann, 2005)
Cell wall-associated proteins			
ECM-binding protein homologue (Ebh)	Anchorless	Binds fibronectin Adheres to endothelial cells	(Clarke & Foster, 2006, Sinha & Herrmann, 2005)

Virulence factor	Secreted/ surface	Function	References
ECM protein-binding protein (Emp)	Anchorless	Binds fibrinogen, fibronectin, and vitronectin Implicated in biofilm formation	(Johnson *et al.*, 2008, Sinha & Herrmann, 2005)
Enolase	Anchorless	Binds laminin Enables *S. aureus* to cross the vasculature	(Clarke & Foster, 2006)
Autolysins (Atl, Aaa)	Anchorless	Bind fibrinogen, fibronectin, vitronectin Atl has been implicated in biofilm formation	(Clarke & Foster, 2006)
Extracellular fibrinogen-binding protein (Efb)	Anchorless	Prevents complement from binding the cell wall Impairs wound healing Binds fibrinogen, inhibiting platelet activation and aggregation	(Foster, 2005, Shannon & Flock, 2004)
Penicillin-binding proteins	Cytoplasmic membrane	Involved in cell wall assembly Confer resistance to penicillinase-resistant penicillins and cephalosporins	(Lowy, 2003)
Elastin binding protein (Ebps)	Trans-membranous	Binds elastin Role in clinical disease unknown	(Downer *et al.*, 2002)

Virulence factor	Secreted/ surface	Function	References
Plasmin-sensitive protein (Pls)	Anchored	Homologue of Sdr proteins and SasG Gene present in type I SCC*mec* May inhibit ClfA and SpA expression Reduces IgG, fibrinogen and fibronectin binding independent of FnBP expression, possibly via steric hindrance Inhibits platelet aggregation and endothelial cell invasion Adheres to lipids, nasal epithelium and keratinocytes	(Clarke & Foster, 2006, Hussain *et al.*, 2009, Juuti *et al.*, 2004, Sinha & Herrmann, 2005)
Serine-rich adhesin for platelets (SraP)	Anchored	Binds platelets Associated with increased virulence in infective endocarditis	(Siboo *et al.*, 2005)
Sas proteins	Anchored	Include SasA, SasC, SasD and SasF to SasH SasC has been implicated in cell aggregation and biofilm formation. SasG inhibits binding of exponential phase MSCRAMMs to plasma proteins and is implicated in biofilm formation Prevalence of genes encoding SasG and SasH are increased in invasive as compared to carriage isolates Functions of other Sas proteins unknown	(Clarke & Foster, 2006, Corrigan *et al.*, 2009, Roche *et al.*, 2003)

Virulence factor	Secreted/ surface	Function	References
Collagen adhesin (Cna)	Anchored	Binds collagen Facilitates thrombus formation under high shear Implicated in septic arthritis, osteomyelitis and later stages of infective endocarditis	(Clarke & Foster, 2006)
FnBPA, FnBPB	Anchored	See section 1.3.5.1	
ClfA, ClfB	Anchored	See sections 1.3.5.2 and 1.3.5.3	
SdrC	Anchored	Binds nasal epithelium	(Corrigan *et al.*, 2009)
SdrD	Anchored	Binds nasal epithelium Implicated in abscess formation and osteomyelitis	(Cheng *et al.*, 2009, Corrigan *et al.*, 2009)
SdrE	Anchored	Induces weak platelet aggregation when expressed on *Lactococcus lactis* (mechanisms unknown)	(O'Brien *et al.*, 2002a)
SpA	Anchored	See section 1.3.5.4	

Virulence factor	Secreted/ surface	Function	References
IsdA and IsdB	Anchored	See Sections 1.3.5.5.1 and 1.3.5.5.2	
IsdC	Anchored	Binds haemin Role in clinical disease unknown	(Mazmanian *et al.*, 2003)
IsdH	Anchored	Binds haptoglobin and haptoglobin-haemoglobin complexes Role in clinical disease unknown	(Dryla *et al.*, 2003)

ARDS, adult respiratory distress syndrome; TSST-1, toxic shock syndrome toxin-1; MHC, major histocompatibility class; TSS, toxic shock syndrome; SIDS, sudden infant death syndrome; CHIPS, chemotaxis-inhibitory protein of staphylococci; SCIN, staphylococcal complement inhibitor; SSL, staphylococcal superantigen-like protein; PSGL-1, P-selectin glycoprotein ligand-1; ICAM-1, intercellular adhesion molecule 1; ECM, extra-cellular matrix; Sdr, serine-aspartate repeat; Sas, *S. aureus* surface protein; SCC*mec*, staphylococcal cassette chromosome *mec*; Clf, clumping factor ; SpA, staphylococcal protein A; FnBP, fibronectin-binding protein; MSCRAMMs, microbial surface components recognising adhesive matrix molecules; Isd, iron-regulated surface determinant.

APPENDIX 2

SUPPLEMENTARY DATA

Table S5.1. Association of GPIIIa $PI^{A1/A2}$ genotype with lag time to *S. aureus*-induced platelet aggregation.

S. aureus strain and growth phase	GPIIIa $PI^{A1/A2}$ genotype			
	A1/A1	A1/A2	A2/A2	P value
Newman, stationary	1.92 ± 0.65 min	1.83 ± 0.47 min	2.63 ± 1.25 min	0.451
209, stationary	8.60 ± 3.51 min	12.44 ± 7.28 min	6.08 ± 1.41 min	0.743
Newman, exponential	6.07 ± 1.41 min	6.60 ± 1.26 min	6.92 ± 1.03 min	0.060
207, exponential	9.19 ± 3.36 min	9.11 ± 3.62 min	8.31 ± 5.14 min	0.868
209, exponential	1.94 ± 0.64 min	1.84 ± 0.50 min	3.13 ± 1.82 min	0.377

Table S5.2. Association of GPIIIa $PI^{A1/A2}$ genotype with rate of platelet aggregation induced by *S. aureus* and pharmacological agonists.

Agonist	GPIIIa $PI^{A1/A2}$ genotype			P value
	A1/A1	A1/A2	A2/A2	
Newman, stationary	34 ± 13	37 ± 10	35 ± 14	0.520
209, stationary	17 ± 5	15 ± 5	13 ± 8	0.709
Newman, exponential	17 ± 7	15 ± 9	20 ± 12	0.481
207, exponential	11 ± 6	13 ± 5	13 ± 6	0.392
209, exponential	29 ± 12	30 ± 11	26 ± 12	0.646
ADP	47 ± 13	49 ± 12	38 ± 10	0.154
Ristocetin	39 ± 12	44 ± 11	51 ± 14	0.146
SFLLRN-NH_2	59 ± 14	62 ± 19	68 ± 14	0.395

Only results where platelet aggregation was induced are included

Table S5.3. Association of GPIIIa $PI^{A1/A2}$ genotype with maximal percentage platelet aggregation induced by *S. aureus* and pharmacological agonists.

Agonist	GPIIIa $PI^{A1/A2}$ genotype			P value
	A1/A1	A1/A2	A2/A2	
Newman, stationary	67 ± 10 %	69 ± 7 %	66 ± 2 %	0.914
209, stationary	68 ± 8 %	64 ± 10 %	63 ± 33 %	0.060
Newman, exponential	69 ± 12 %	60 ± 19 %	76 ± 7 %	0.106
207, exponential	66 ± 15 %	66 ± 13 %	70 ± 16 %	0.515
209, exponential	63 ± 9 %	63 ± 14 %	67 ± 4 %	0.461
ADP	69 ± 12 %	74 ± 12 %	70 ± 6 %	0.153
Ristocetin	81 ± 7 %	81 ± 8 %	81 ± 10 %	0.955
SFLLRN-NH_2	68 ± 12 %	69 ± 13 %	69 ± 7 %	0.929

Only results where platelet aggregation was induced are included

Table S5.4. Association of GPIIIa $PI^{A1/A2}$ genotype with lag time to *S. aureus*-induced aggregation of washed platelets.

S. aureus strain and growth phase	GPIIIa $PI^{A1/A2}$ genotype			
	A1/A1	A1/A2	A2/A2	P value
Newman, stationary	3.00 ± 0.90 min	2.89 ± 1.26 min	9.21 ± 10.56 min	0.034
209, stationary	10.20 ± 8.73 min	9.10 ± 9.18 min	13.66 ± 7.88 min	0.342
Newman, exponential	8.65 ± 8.02 min	9.05 ± 9.16 min	21.01 ± 7.99 min	0.059
207, exponential	25 min *	19.24 ± 8.78 min	22.93 ± 4.14 min	0.047
209, exponential	7.74 ± 5.48 min	8.74 ± 7.43 min	16.32 ± 10.10 min	0.176

* Absence of platelet aggregation

Table S5.5. Association of GPIIIa $PI^{A1/A2}$ genotype with rate of *S. aureus*-induced aggregation of washed platelets.

Agonist	GPIIIa $PI^{A1/A2}$ genotype			P value
	A1/A1	A1/A2	A2/A2	
Newman, stationary	50 ± 13	50 ± 13	46 ± 10	0.817
209, stationary	29 ± 13	41 ± 9	19 ± 18	0.042
Newman, exponential	22 ± 13	29 ± 14	N/A	0.505
209, exponential	28 ± 12	29 ± 12	34 ± 14	0.778
ADP	30 ± 24	30 ± 17	17 ± 18	0.533
Ristocetin	28 ± 11	26 ± 6	23 ± 5	0.500
SFLLRN-NH_2	52 ± 25	61 ± 18	79 ± 51	0.374

Only results where platelet aggregation was induced are included

Table S5.6. Association of GPIIIa $PI^{A1/A2}$ genotype with percentage *S. aureus*-induced aggregation of washed platelets.

Agonist	GPIIIa $PI^{A1/A2}$ genotype			P value
	A1/A1	A1/A2	A2/A2	
Newman, stationary	82 ± 4 %	80 ± 6 %	78 ± 5 %	0.431
209, stationary	73 ± 12 %	77 ± 7 %	73 ± 17 %	0.601
Newman, exponential	66 ± 16 %	75 ± 6 %	N/A	0.408
209, exponential	71 ± 12 %	72 ± 8 %	75 ± 1 %	0.928
ADP	29 ± 20 %	36 ± 17 %	12 ± 16 %	0.062
Ristocetin	67 ± 7 %	70 ± 7 %	73 ± 6 %	0.273
SFLLRN-NH_2	71 ± 19 %	75 ± 5 %	64 ± 22 %	0.622

Only results where platelet aggregation was induced are included

Table S5.7. Association of GPIIIa $PI^{A1/A2}$ genotype with percentage of platelets bound to *S. aureus*.

S. aureus strain and growth phase	GPIIIa $PI^{A1/A2}$ genotype			
	A1/A1	A1/A2	A2/A2	P value
Newman, stationary	84 ± 21 %	85 ± 26 %	91 ± 8 %	0.928
209, stationary	86 ± 22 %	86 ± 23 %	92 ± 8 %	0.989
Newman, exponential	58 ± 27 %	53 ± 26 %	70 ± 31 %	0.516
207, exponential	78 ± 25 %	74 ± 28 %	77 ± 20 %	0.745
209, exponential	57 ± 25 %	52 ± 28 %	58 ± 19 %	0.808

Table S5.8. Association of GPIIIa $PI^{A1/A2}$ genotype with percentage of *S. aureus* cells bound to platelets.

S. aureus strain and growth phase	GPIIIa $PI^{A1/A2}$ genotype			
	A1/A1	**A1/A2**	**A2/A2**	**P value**
Newman, stationary	49 ± 10 %	44 ± 15 %	56 ± 9 %	0.318
209, stationary	44 ± 12 %	38 ± 12 %	48 ± 12 %	0.257
Newman, exponential	22 ± 12 %	18 ± 11 %	25 ± 8 %	0.508
207, exponential	24 ± 10 %	21 ± 8 %	23 ± 3 %	0.566
209, exponential	18 ± 11 %	14 ± 7 %	17 ± 3 %	0.669

Table S5.9. Association of GPIIIa $PI^{A1/A2}$ genotype with absolute levels of P-selectin expression induced by *S. aureus* and SFLLRN-NH_2.

Agonist	GPIIIa $PI^{A1/A2}$ genotype			P value
	A1/A1	A1/A2	A2/A2	
Newman, stationary	55 ± 29 %	49 ± 25 %	43 ± 33 %	0.842
209, stationary	26 ± 11 %	18 ± 11 %	24 ± 6 %	0.191
Newman, exponential	36 ± 20 %	40 ± 19 %	31 ± 16 %	0.709
207, exponential	24 ± 15 %	18 ± 12 %	25 ± 11 %	0.562
209, exponential	28 ± 17 %	20 ± 12 %	21 ± 4 %	0.282
SFLLRN-NH_2	82 ± 14 %	83 ± 6 %	78 ± 14 %	0.730

Table S5.10. Association of GPIb Kozak sequence genotype with lag time to *S. aureus*-induced platelet aggregation.

	GPIb Kozak sequence genotype		
***S. aureus* strain and growth phase**	**T/T**	**T/C**	**P value**
Newman, stationary	1.94 ± 0.65 min	2.01 ± 0.88 min	0.898
209, stationary	8.79 ± 4.20 min	8.78 ± 3.19 min	0.952
Newman, exponential	6.09 ± 1.37 min	6.65 ± 1.24 min	0.021
207, exponential	9.23 ± 3.33 min	9.20 ± 3.62 min	0.775
209, exponential	2.05 ± 1.01 min	1.99 ± 0.78 min	0.678

Table S5.11. Association of GPIb Kozak sequence genotype with rate of platelet aggregation induced by *S. aureus* and pharmacological agonists.

	GPIb Kozak sequence genotype		
Agonist	**T/T**	**T/C**	**P value**
Newman, stationary	35 ± 11	35 ± 13	0.851
209, stationary	14 ± 6	14 ± 4	0.706
Newman, exponential	17 ± 9	15 ± 6	0.186
207, exponential	12 ± 6	12 ± 5	0.613
209, exponential	29 ± 12	30 ± 11	0.494
ADP	47 ± 11	48 ± 14	0.914
Ristocetin	40 ± 12	41 ± 12	0.988
SFLLRN-NH_2	61 ± 16	60 ± 14	0.978

Only results where platelet aggregation was induced are included

Table S5.12. Association of GPIb Kozak sequence genotype with maximal percentage platelet aggregation induced by *S. aureus* and pharmacological agonists.

	GPIb Kozak sequence genotype		
Agonist	**T/T**	**T/C**	**P value**
Newman, stationary	67 ± 8 %	67 ± 10 %	0.313
209, stationary	64 ± 12 %	69 ± 8 %	0.033
Newman, exponential	67 ± 16 %	67 ± 12 %	0.400
207, exponential	64 ± 17 %	66 ± 12 %	0.878
209, exponential	62 ± 10 %	63 ± 10 %	0.700
ADP	71 ± 12 %	69 ± 13 %	0.911
Ristocetin	82 ± 7 %	80 ± 7 %	0.246
SFLLRN-NH_2	69 ± 12 %	65 ± 14 %	0.258

Only results where platelet aggregation was induced are included

Table S5.13. Association of GPIb Kozak sequence genotype with lag time to *S. aureus*-induced aggregation of washed platelets.

S. aureus strain and growth phase	GPIb Kozak sequence genotype		
	T/T	T/C	P value
Newman, stationary	4.21 ± 5.15 min	3.08 ± 0.96 min	0.747
209, stationary	9.98 ± 8.36 min	10.95 ± 9.34 min	0.948
Newman, exponential	8.31 ± 7.67 min	14.07 ± 10.59 min	0.328
207, exponential	22.27 ± 6.56 min	24.25 ± 2.49 min	0.536
209, exponential	7.70 ± 5.55 min	11.75 ± 8.94 min	0.332

Table S5.14. Association of GPIb Kozak sequence genotype with rate of *S. aureus*-induced aggregation of washed platelets.

	GPIb Kozak sequence genotype		
Agonist	**T/T**	**T/C**	**P value**
Newman, stationary	49 ± 13	51 ± 11	0.839
209, stationary	30 ± 15	35 ± 12	0.457
Newman, exponential	23 ± 13	27 ± 14	0.417
209, exponential	28 ± 13	30 ± 8	0.956
ADP	25 ± 21	38 ± 19	0.059
Ristocetin	26 ± 6	28 ± 13	0.880
SFLLRN-NH_2	54 ± 23	66 ± 36	0.547

Only results where platelet aggregation was induced are included

Table S5.15. Association of GPIb Kozak sequence genotype with maximal percentage *S. aureus*-induced aggregation of washed platelets.

	GPIb Kozak sequence genotype		
Agonist	**T/T**	**T/C**	**P value**
Newman, stationary	81 ± 6 %	81 ± 3 %	0.840
209, stationary	72 ± 13 %	78 ± 5 %	0.653
Newman, exponential	69 ± 14 %	72 ± 15 %	0.356
209, exponential	71 ± 13 %	74 ± 3 %	0.911
ADP	30 ± 21 %	26 ± 18 %	0.730
Ristocetin	70 ± 7 %	67 ± 6 %	0.244
SFLLRN-NH_2	69 ± 12 %	73 ± 8 %	0.490

Only results where platelet aggregation was induced are included

Table S5.16. Association of GPIb Kozak sequence genotype with percentage of platelets bound to *S. aureus*.

S. aureus strain and growth phase	GPIb Kozak sequence genotype		
	T/T	T/C	P value
Newman, stationary	86 ± 21 %	84 ± 22 %	0.561
209, stationary	88 ± 22 %	85 ± 20 %	0.989
Newman, exponential	61 ± 32 %	54 ± 25 %	0.516
207, exponential	80 ± 25 %	72 ± 25 %	0.745
209, exponential	58 ± 28 %	50 ± 16 %	0.808

Table S5.17. Association of GPIb Kozak sequence genotype with percentage of *S. aureus* bound to platelets.

	GPIb Kozak sequence genotype		
***S. aureus* strain and growth phase**	**T/T**	**T/C**	**P value**
Newman, stationary	47 ± 13 %	50 ± 11 %	0.747
209, stationary	41 ± 13 %	45 ± 11 %	0.401
Newman, exponential	21 ± 13 %	21 ± 8 %	0.813
207, exponential	23 ± 10 %	23 ± 4 %	0.983
209, exponential	17 ± 11 %	17 ± 4 %	0.566

Table S5.18. Association of GPIb Kozak sequence genotype with absolute levels of P-selectin expression induced by *S. aureus* and SFLLRN-NH_2.

	GPIb Kozak sequence genotype		
Agonist	**T/T**	**T/C**	**P value**
Newman, stationary	49 ± 25 %	57 ± 31 %	0.417
209, stationary	24 ± 12 %	23 ± 8 %	0.808
Newman, exponential	38 ± 20 %	34 ± 18 %	0.776
207, exponential	23 ± 16 %	21 ± 8 %	1.000
209, exponential	25 ± 16 %	24 ± 12 %	1.000
SFLLRN-NH_2	84 ± 8 %	78 ± 16 %	0.378

Table S5.19. Association of GPIb HPA-2 genotype with lag time to *S. aureus*-induced platelet aggregation.

S. aureus strain and growth phase	GPIb HPA-2 genotype			
	2a/2a	2a/2b	2b/2b *	P value
Newman, stationary	1.96 ± 0.78 min	1.98 ± 0.62 min	2.41 min	0.603
209, stationary	8.76 ± 4.14 min	8.97 ± 2.88 min	6.89 min	0.746
Newman, exponential	6.37 ± 1.36 min	5.96 ± 1.30 min	7.17 min	0.394
207, exponential	9.10 ± 3.47 min	9.65 ± 3.37 min	9.02 min	0.755
209, exponential	2.01 ± 0.92 min	1.95 ± 0.65 min	2.95 min	0.364

* One individual had the HPA-2b/2b genotype

Table S5.20. Association of GPIb HPA-2 genotype with rate of platelet aggregation induced by *S. aureus* and pharmacological agonists.

	GPIb HPA-2 genotype			
Agonist	**2a/2a**	**2a/2b**	**2b/2b ***	**P value**
Newman, stationary	35 ± 11	34 ± 13	49	0.402
209, stationary	14 ± 5	15 ± 5	15	0.371
Newman, exponential	16 ± 8	18 ± 9	20	0.261
207, exponential	12 ± 5	13 ± 6	14	0.577
209, exponential	29 ± 12	28 ± 13	49	0.253
ADP	49 ± 13	43 ± 11	41	0.142
Ristocetin	40 ± 12	42 ± 14	35	0.127
SFLLRN-NH_2	61 ± 15	59 ± 17	65	0.310

* One individual had the HPA-2b/2b genotype

Only results where platelet aggregation was induced are included

Table S5.21. Association of GPIb HPA-2 genotype with maximal percentage platelet aggregation induced by *S. aureus* and pharmacological agonists.

	GPIb HPA-2 genotype			
Agonist	**2a/2a**	**2a/2b**	**2b/2b ***	**P value**
Newman, stationary	67 ± 9 %	67 ± 8 %	74 %	0.553
209, stationary	65 ± 11 %	69 ± 9 %	77 %	0.304
Newman, exponential	67 ± 15 %	68 ± 11 %	70 %	0.883
207, exponential	64 ± 16 %	66 ± 15 %	75 %	0.640
209, exponential	62 ± 11 %	66 ± 8 %	62 %	0.502
ADP	70 ± 12 %	73 ± 13 %	67 %	0.354
Ristocetin	81 ± 7 %	80 ± 8 %	70 %	0.668
SFLLRN-NH_2	68 ± 12 %	68 ± 16 %	50 %	0.836

* One individual had the HPA-2b/2b genotype

Only results where platelet aggregation was induced are included

Table S5.22. Association of GPIb variable number of tandem repeat (VNTR) genotype with lag time to *S. aureus*-induced platelet aggregation.

S. aureus strain and growth phase	GPIb VNTR genotype						
	A/D*	B/C	B/D	C/C	C/D	D/D*	P value
Newman, stationary	2.76 min	1.96 ± 0.64 min	1.88 ± 0.11 min	1.92 ± 0.78 min	2.22 ± 0.79 min	1.22 min	0.487
209, stationary	9.27 min	9.05 ± 2.64 min	8.51 ± 4.25 min	8.78 ± 4.45 min	8.46 ± 2.88 min	7.97 min	1.000
Newman, exponential	8.27 min	6.17 ± 1.22 min	5.51 ± 1.40 min	6.43 ± 1.38 min	5.81 ± 1.32 min	5.30 min	0.395
207, exponential	11.26 min	8.52 ± 3.25 min	13.76 ± 7.23 min	9.18 ± 3.51 min	9.56 ± 2.49 min	9.18 min	0.725
209, exponential	2.52 min	1.96 ± 0.71 min	2.00 ± 0.38 min	2.05 ± 0.99 min	1.93 ± 0.49 min	1.22 min	0.867

* A/D and D/D genotypes were present in one individual each

Table S5.23. Association of GPIb variable number of tandem repeat (VNTR) genotype with rate of platelet aggregation induced by *S. aureus* and pharmacological agonists.

	GPIb VNTR genotype						
Agonist	**A/D***	**B/C**	**B/D**	**C/C**	**C/D**	**D/D***	**P value**
Newman, stationary	22	35 ± 14	46 ± 2	36 ± 11	31 ± 15	34	0.324
209, stationary	16	15 ± 4	22 ± 4	14 ± 6	13 ± 4	10	0.233
Newman, exponential	4	19 ± 7	22 ± 18	15 ± 8	17 ± 9	17	0.314
207, exponential	8	13 ± 6	17 ± 2	11 ± 5	12 ± 6	10	0.282
209, exponential	19	30 ± 11	47 ± 14	29 ± 12	29 ± 13	30	0.375
ADP	33	43 ± 11	46 ± 3	49 ± 12	47 ± 15	45	0.323
Ristocetin	42	41 ± 13	59 ± 8	40 ± 12	39 ± 9	46	0.382
SFLLRN-NH_2	46	62 ± 18	58 ± 16	62 ± 16	55 ± 13	49	0.512

* A/D and D/D genotypes were present in one individual each

Only results where platelet aggregation was induced are included

Table S5.24. Association of GPIb variable number of tandem repeat (VNTR) genotype with maximal percentage platelet aggregation induced by *S. aureus* and pharmacological agonists.

	GPIb VNTR genotype						
Agonist	**A/D***	**B/C**	**B/D**	**C/C**	**C/D**	**D/D***	**P value**
Newman, stationary	64 %	67 ± 8 %	74 ± 3 %	68 ± 9 %	62 ± 13 %	61 %	0.476
209, stationary	77 %	68 ± 8 %	83 ± 7 %	64 ± 12 %	70 ± 9 %	61 %	0.030
Newman, exponential	43 %	69 ± 9 %	73 ± 14 %	68 ± 15 %	61 ± 16 %	54 %	0.104
207, exponential	67 %	66 ± 16 %	71 ± 4 %	65 ± 15 %	63 ± 20 %	61 %	0.708
209, exponential	64 %	66 ± 6 %	65 ± 4 %	62 ± 11 %	60 ± 14 %	55 %	0.326
ADP	51 %	73 ± 13 %	78 ± 7 %	70 ± 11 %	69 ± 16 %	77 %	0.412
Ristocetin	88 %	77 ± 6 %	89 ± 1 %	82 ± 8 %	83 ± 7 %	73 %	0.016
SFLLRN-NH_2	73 %	66 ± 16 %	79 ± 5 %	69 ± 11 %	63 ± 16 %	60 %	0.293

* A/D and D/D genotypes were present in one individual each

Only results where platelet aggregation was induced are included

Table S5.25. Association of FcγRIIa H131R genotype with lag time to *S. aureus*-induced platelet aggregation.

S. aureus strain and growth phase	FcγRIIa H131R genotype			
	H/H	H/R	R/R	P value
Newman, stationary	1.82 ± 0.58 min	1.94 ± 0.68 min	2.02 ± 0.69 min	0.384
209, stationary	8.02 ± 3.69 min	8.31 ± 3.85 min	9.91 ± 4.00 min	0.287
Newman, exponential	5.90 ± 1.61 min	6.67 ± 1.29 min	5.92 ± 1.07 min	0.077
207, exponential	9.28 ± 3.64 min	9.18 ± 3.60 min	8.87 ± 3.23 min	0.765
209, exponential	1.86 ± 0.51 min	1.92 ± 0.64 min	2.16 ± 1.01 min	0.401

Table S5.26. Association of FcγRIIa H131R genotype with rate of platelet aggregation induced by *S. aureus* and pharmacological agonists.

	FcγRIIa H131R genotype			
Agonist	**H/H**	**H/R**	**R/R**	**P value**
Newman, stationary	37 ± 10	36 ± 11	31 ± 14	0.244
209, stationary	15 ± 4	15 ± 5	11 ± 5	0.038
Newman, exponential	16 ± 6	16 ± 9	16 ± 7	0.945
207, exponential	13 ± 5	12 ± 6	11 ± 5	0.355
209, exponential	31 ± 9	31 ± 12	25 ± 14	0.207
ADP	45 ± 11	47 ± 13	50 ± 13	0.745
Ristocetin	43 ± 14	39 ± 13	41 ± 9	0.452
SFLLRN-NH_2	59 ± 15	62 ± 18	61 ± 14	0.275

Only results where platelet aggregation was induced are included

Table S5.27. Association of FcγRIIa H131R genotype with maximal percentage platelet aggregation induced by *S. aureus* and pharmacological agonists.

	FcγRIIa H131R genotype			
Agonist	**H/H**	**H/R**	**R/R**	**P value**
Newman, stationary	69 ± 9 %	68.0 ± 8%	65 ± 10 %	0.351
209, stationary	68 ± 9 %	67 ± 10%	64 ± 14 %	0.611
Newman, exponential	70 ± 14 %	65 ± 17 %	66 ± 9 %	0.285
207, exponential	69 ± 10 %	64 ± 17 %	66 ± 13 %	0.576
209, exponential	65 ± 6 %	65 ± 8 %	57 ± 15 %	0.043
ADP	73 ± 11 %	70 ± 13 %	69 ± 11 %	0.206
Ristocetin	82 ± 7 %	82 ± 7 %	80 ± 8 %	0.729
SFLLRN-NH_2	65 ± 15 %	70 ± 12 %	68 ± 9 %	0.275

Only results where platelet aggregation was induced are included

Table S5.28. Association of FcγRIIa H131R genotype with lag time to *S. aureus*-induced aggregation of washed platelets.

***S. aureus* strain and growth phase**	**FcγRIIa H131R genotype**			
	H/H	**H/R**	**R/R**	**P value**
Newman, stationary	3.26 ± 0.66 min	3.19 ± 1.18 min	5.42 ± 7.96 min	0.949
209, stationary	9.00 ± 8.18 min	9.59 ± 8.13 min	12.81 ± 10.31 min	0.948
Newman, exponential	11.98 ± 10.44 min	8.74 ± 8.19 min	12.61 ± 10.42 min	0.328
207, exponential	23.62 ± 3.38 min	22.85 ± 5.98 min	22.82 ± 6.18 min	0.536
209, exponential	9.57 ± 7.90 min	9.29 ± 7.23 min	8.68 ± 7.26 min	0.332

Table S5.29. Association of FcγRIIa H131R genotype with rate of *S. aureus*-induced aggregation of washed platelets.

	FcγRIIa H131R genotype			
Agonist	**H/H**	**H/R**	**R/R**	**P value**
Newman, stationary	55 ± 9	49 ± 14	47 ± 12	0.616
209, stationary	32 ± 11	31 ± 17	32 ± 10	0.948
Newman, exponential	25 ± 17	27 ± 12	16 ± 12	0.248
209, exponential	36 ± 7	28 ± 13	25 ± 12	0.171
ADP	31 ± 16	31 ± 25	23 ± 12	0.738
Ristocetin	32 ± 17	25 ± 6	27 ± 5	0.834
SFLLRN-NH_2	67 ± 36	52 ± 26	62 ± 28	0.603

Only results where platelet aggregation was induced were included

Table S5.30. Association of FcγRIIa H131R genotype with maximal percentage aggregation of washed platelets induced by *S. aureus* and pharmacological agonists.

	FcγRIIa H131R genotype			
Agonist	**H/H**	**H/R**	**R/R**	**P value**
Newman, stationary	81 ± 3 %	79 ± 5 %	85 ± 4 %	0.066
209, stationary	75 ± 6 %	73 ± 14 %	77 ± 8 %	0.385
Newman, exponential	69 ± 5 %	75 ± 5 %	56 ± 22 %	0.207
209, exponential	76 ± 3 %	69 ± 13 %	74 ± 5 %	0.251
ADP	33 ± 31 %	29 ± 16 %	26 ± 19 %	0.877
Ristocetin	67 ± 6 %	68 ± 7 %	72 ± 7 %	0.520
SFLLRN-NH_2	75 ± 7 %	71 ± 7 %	68 ± 18 %	0.622

Only results where platelet aggregation was induced were included

Table S5.31. Association of FcγRIIa H131R genotype with binding of platelets to *S. aureus*

S. aureus strain and growth phase	FcγRIIa H131R genotype			
	H/H	H/R	R/R	P value
Newman, stationary	87 ± 23 %	87 ± 18 %	80 ± 26 %	0.456
209, stationary	84 ± 28 %	90 ± 16 %	82 ± 25 %	0.544
Newman, exponential	56 ± 30 %	60 ± 29 %	57 ± 34 %	0.964
207, exponential	75 ± 31 %	81 ± 21 %	70 ± 27 %	0.476
209, exponential	51 ± 27 %	59 ± 23 %	51 ± 30 %	0.730

Table S5.32. Association of FcγRIIa H131R genotype with binding of *S. aureus* to platelets

S. aureus strain and growth phase	FcγRIIa H131R genotype			
	H/H	H/R	R/R	P value
Newman, stationary	52 ± 9 %	48 ± 13 %	47 ± 14 %	0.768
209, stationary	47 ± 13 %	42 ± 12 %	40 ± 12 %	0.550
Newman, exponential	21 ± 6 %	22 ± 14 %	19 ± 9 %	0.896
207, exponential	20 ± 6 %	25 ± 10 %	22 ± 7 %	0.699
209, exponential	17 ± 1 %	18 ± 11 %	15 ± 5 %	0.591

Table S5.33. Association of FcγRIIa H131R genotype with *S. aureus*-induced platelet activation

Agonist	FcγRIIa H131R genotype			P value
	H/H	H/R	R/R	
Newman, stationary	64 ± 29 %	54 ± 27 %	40 ± 25 %	0.294
209, stationary	27 ± 12 %	22 ± 12 %	23 ± 9 %	0.834
Newman, exponential	49 ± 24 %	38 ± 16 %	25 ± 15 %	0.059
207, exponential	31 ± 22 %	19 ± 11 %	22 ± 10 %	0.450
209, exponential	34 ± 23 %	23 ± 14 %	21 ± 6 %	0.410
SFLLRN-NH_2	78 ± 23 %	84 ± 6 %	79 ± 11 %	0.473

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